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Benzodiazepines and the $GABA_A$ Receptor: Effects of Chronic Exposure

by

Michelle Ingird Arnot ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Pharmacology

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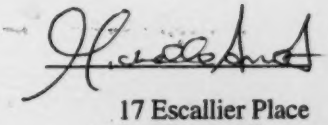

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A mind once stretched by a new idea,
never regains its original dimensions.

— Author Unknown

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Benzodiazepines and the $GABA_A$ receptor: effects of chronic exposure by Michelle Ingrid Arnot in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The benzodiazepine pharmacological profile includes sedation, anxiolysis, muscle relaxation and anticonvulsant effects; however, after long term treatment with full agonists tolerance develops to certain aspects of this profile. These compounds produce their overt effects via allosteric modulation of the $GABA_A$ receptor. The $GABA_A$ receptor is a oligomeric pentamer surrounding a central chloride ion pore; activation of the receptor normally results in chloride influx and membrane hyperpolarization. This pentameric complex is comprised of different subunits, each of which are encoded by a separate gene.

The experiments outlined in this thesis attempt to increase our understanding of the response of the GABAergic system to chronic benzodiazepine exposure. An alternative mechanism for drug delivery was established to ensure continuous diazepam delivery over an extended period. This paradigm was then utilized for exposure of rats for 7, 14, 28 days continuous infusion of 15 mg/kg/day diazepam. There were temporal alterations in steady-state $GABA_A$ receptor mRNA levels which were brain region specific, and either attenuated or rebounded after 3 days drug cessation. This continuous infusion was compared to a daily injection regime; each 14 day dosing paradigm resulted in equivalent daily doses of 15 mg/kg diazepam, yet there were differential alterations in cortical $GABA_A$ receptor steady-state mRNA levels. Both chronic dosing regimes resulted in reduced GABA enhancement of [^{3}H]-flunitrazepam binding, but animals from the daily diazepam injection regime exhibited an increase in cortical [^{3}H]-Ro 15-4513 binding and in the proportion of diazepam insensitive binding. Following each diazepam administration there was a similar development of tolerance to sedation, yet there were differences in the development of anxiolytic tolerance. Alterations in the $GABA_A$ receptor after 14 days of daily diazepam injection were compared to similar treatment with partial agonists, which do not result in tolerance development. Diazepam chronic treatment demonstrated changes in steady-state mRNA levels which were distinct from partial agonist induced alterations. These data are consistent with the hypothesis that benzodiazepine chronic administration

results in the concomitant up- and down-regulation of $GABA_A$ receptor subunit gene expression. This "isoform switching" subsequently alters benzodiazepine binding profiles and modulation between the GABA and benzodiazepine-sites in an attempt to maintain homeostatic balance.

To my parents John and Linda,

my brother Jon,

and in loving memory of my grandmother

Susie L. Kvamme

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ANCOVA	analysis of covariance
BSA	bovine serum albumin
BZ	benzodiazepine
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
DIS	diazepam insensitive
DMCM	6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester
DMSO	dimethyl sulfoxide
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
GABA	γ-aminobutyric acid
GlyR	glycine receptor
HEK	human embryonic kidney cells
HPLC	high pressure liquid chromatography
i.p.	intraperitoneal
LGIC	ligand gated ion channel
mRNA	messenger ribonucleic acid
nAchR	nicotinic acetylcholine receptor
NIH	National Institiutes of Health
NIMH	National Institute of Mental Health
$5HT_3R$	5-hydroxytryptamine/serotonin type 3 receptor
PIPES	piperazine-N,N-bis(2-ethanesulfonic acid)
PCR	polymerase chain reaction
PG	propylene glycol

PKA	protein kinase A
PKC	protein kinase C
p.o.	*per os* (oral)
PTZ	pentylenetetrazole
RNA	ribonucleic acid
s.c.	subcutaneous
SEM	standard error of mean
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

CHAPTER 1

Introduction

General Overview

The serendipitous discovery of chlordiazepoxide (Librium®) and its marketing as an anxiolytic in 1960 began an era of benzodiazepine development and their use as anxiolytics and sedative hypnotics. This class of compounds also exhibited muscle relaxant and anticonvulsant effects, and with the development of diazepam (Valium®), became one of the most prescribed classes of drugs in the late 1970's to the mid 1980's. However, it was not until nearly 20 years after their discovery that the primary mechanism of action of the benzodiazepines became known. It was found that benzodiazepines potentiate the effects of γ-aminobutyric acid (GABA), the major inhibitory transmitter in the central nervous system (CNS). GABA binds to three different receptors in the CNS, and activation of these receptors results in three distinct responses. The fast responses to GABA occur via the $GABA_A$ receptor where the binding site is linked to a chloride ionophore and binding results in chloride influx and, primarily, membrane hyperpolarization. The slow inhibitory response to GABA results from binding and activation of the $GABA_B$ receptor, which is a G-protein-linked receptor and activation is linked to alterations in Ca^{++} and K^+ channel conductances. The third receptor, the $GABA_C$ receptor, is similar to the $GABA_A$ receptor, with the exception that it is not modulated by benzodiazepines and is primarily found in the retina.

The benzodiazepines recognize the $GABA_A$ receptor, and potentiate the effects of GABA in an allosteric manner. There are binding sites on the $GABA_A$ receptor for other modulatory agents, including the barbiturates, ethanol, some general anesthetics, and the neurosteroids. The $GABA_A$ receptor is a member of the ligand gated ion channel (LGIC) superfamily, other members of which include the nicotinic acetylcholine receptor (nAchR), the glycine receptor (GlyR) and the 5-hydroxytryptamine type 3 ($5HT_3$) receptor. The $GABA_A$ receptor is a heteromeric pentamer surrounding a central chloride ion pore. The pentamer itself is composed of at least 3 different subunits, and currently there have been 7

subunit classes identified: α, β, γ, δ, ε, π, and ρ. However, the ρ subunit is believed to form the $GABA_C$ receptor. As a further complication, many subunit classes are comprised of multiple isoforms, each of which is a separate gene product (α1-6, β1-3, and γ1-3). Therefore, the pentameric combination of the $GABA_A$ receptor may not only vary in the class of subunit, but may also vary with respect to the specific subunit isoform within the receptor complex.

The general intent of the experiments described in this thesis was to gain insight into the interactions between the benzodiazepines and the $GABA_A$ receptor, and specifically into how long-term benzodiazepine exposure alters the homeostatic balance of the GABAergic system. Receptors in general have evolved a number of mechanisms to adapt to alterations in modulatory input, and the $GABA_A$ receptor is no exception. Potential processes for maintaining homeostatic integrity following prolonged ligand exposure include receptor desensitization, allosteric uncoupling between modulatory sites, receptor down-regulation and receptor internalization. Phosphorylation of the receptor may be utilized as a separate mechanism for maintaining this balance or may be implicated with the previously listed methods of homeostatic preservation.

Coordinated regulation of the $GABA_A$ receptor subunits, which combine to form the receptor complex, is a process which is currently unique to this sytem; however, there is increasing evidence that this complex regulatory control may be a common mechansim for the LGIC receptors. *In vitro* expression studies have shown that the pharmacology and the physiology of the $GABA_A$ receptor are dependent upon the specific subunit composition. The benzodiazepine affinity and efficacy may be altered by the various α and γ subunit isoforms; further, activation and desensitization kinetics may also be affected depending on which isoforms are expressed.

If $GABA_A$ receptor subtypes can be altered in response to long-term drug exposure, the manner in which the subsequent receptor interacts with modulatory agents may also be changed. The actions of benzodiazepines are exquistively dependent on $GABA_A$ receptor

subtype. The remodeling of the $GABA_A$ receptor could produce a receptor which is less responsive to the effects of diazepam via the following potential adaptations: (1) a decreased ability to allosterically modulate GABA potentiation, (2) a decreased affinity for benzodiazepines (3) a change in the affinity/efficacy for the GABA agonist or (4) altered receptor kinetics. Another consequence of receptor subtype switching is the reduced ability of the receptor to be involved with neuronal activation and signaling *in vivo*. It is these changes which may underlie behavioral tolerance, attributable to the benzodiazepines being no longer effective at potentiating the effects of GABA, for any of the above reasons.

The clinical relevance of this study relates to the development of behavioral tolerance and the dependence liability with corresponding changes in GABAergic neuroadaptation. The alterations in behavioral activity may be a result of changes in the $GABA_A$ receptor after chronic treatment, and it is possible to correlate, through a number of methods, the behavioral manifestations of tolerance to the alterations in $GABA_A$ receptor steady-state mRNA levels and subsequent benzodiazepine binding characteristics.

The History and Development of Benzodiazepines

The history of the benzodiazepines began with the search for novel tranquilizers in the 1950s. The derivitization of the benzheptoxdiazines by Hoffmann La Roche generated a 1,4-benzodiazepine with unique pharmacological properties based on the behavioral tests utilized for screening new tranquilizers. This chlordiazepoxide compound caused sedation and muscle relaxation, exhibited anticonvulsant properties and had a pronounced taming effect on animals, indicating anxiolytic effects. In 1960, this compound was first marketed as the anxiolytic, Librium®. Derivatives of this parent compound resulted in the development of a family of compounds known as the classical benzodiazepines. These 1,4-benzodiazepines exhibited a spectrum of pharmacokinetic properties, and therefore varied in their suitability to be used as therapeutic agents (Rabow et al., 1995; Sieghart, 1995; Doble and Martin, 1996). For instance, diazepam, which has an extended half-life in man is primarily used as an anxiolytic because of its prolonged effect, whereas triazolam has a short half-life and is therefore used as a hypnotic because it does not cause next day drowsiness (the hang-over effect).

The mechanisms of action of benzodiazepines were not understood until it was established that diazepam potentiated the dorsal root potential in cat spinal cord (Schmidt, 1971), and this later became associated with the inhibitory circuits which use GABA as the transmitter substance (Levy 1977). GABA itself was first known as Factor 1, when in 1957, it was isolated from cow brain and *in vitro* application resulted in the inhibition of spontaneous discharges from the crayfish stretch receptor neuron (Bazemore et al., 1957). The effects of benzodiazepines at postsynaptic receptors were shown to be dependent on the presence of GABA (Polc et al., 1974; Polc and Haefely, 1976; Study and Barker, 1981) and, like GABA and synthetic GABA-site agonists, decreased cerebellar cGMP levels (Costa et al., 1975, 1978). High affinity benzodiazepine binding sites in rat brain membranes were not identified until the development of radioligand binding assays, which

labeled these sites with [^{3}H]-diazepam (Squires and Braestrup, 1977; Mohler and Okada, 1977). The high affinity binding of [^{3}H]-diazepam and [^{3}H]-flunitrazepam were displaced by other benzodiazepines (Duka et al., 1979), yet GABA-site agonists and antagonists failed to alter this radiolabeled benzodiazepine binding. Further, this population of high affinity diazepam binding sites was co-localized with $GABA_A$ receptors. The mounting evidence that benzodiazepines modified [^{3}H]-GABA binding *in vitro* indicated that although the GABA and benzodiazepine binding sites were separate, they may be coupled in some manner (Briley and Langer, 1978; Martin and Candy, 1978; Tallman et al., 1978). Further *in vivo* autoradiographic studies demonstrated similar patterns of anatomical localization of [^{3}H]-benzodiazepine, [^{3}H]-GABA, and [^{3}H]-muscimol binding (Young and Kuhar, 1979; Penny et al., 1981). The correlation between benzodiazepine potency and affinity for the labeled benzodiazepine site led to the conclusion that benzodiazepines exert their clinical actions via the $GABA_A$ receptor (Haefely et al., 1985).

Benzodiazepines were shown to enhance GABA's actions by increasing the frequency of single channel openings (Study and Barker, 1981), which was dissimilar to the barbiturates which prolonged the mean channel open time and can directly activate the $GABA_A$ receptor. At a given GABA concentration the benzodiazepines increased the enhancement of the receptor's affinity for GABA, therefore shifting the dose-response curve to the left (Skerritt and MacDonald, 1984). Recently, the benzodiazepines have been shown to increase the occurrence of burst activity, without altering the duration of this activity (MacDonald and Twyman, 1992; MacDonald and Olsen, 1994). The observed changes in channel behavior may be explained as a change in the agonist association rate and as a change in desensitization kinetics (Lavoie and Twyman, 1996).

Radiolabeled binding assays revealed two subtypes of benzodiazepine sites, based on the affinity for CL 218872 in different regions of the brain (Braestrup et al., 1979; Nielsen and Braestrup, 1980) and these became known as BZ1 and BZ2 receptor subtypes (Lippa et al., 1979a,b). In recent years, this division into subtypes has become far more

complicated with the explosion of information regarding the molecular biology of the $GABA_A$ receptor. Using benzodiazepine high affinity chromatography, co-purification of the benzodiazepine and muscimol binding sites led to the isolation of the $GABA_A$ receptor and the initial purification of the first α and β subunits (Sigel et al, 1983; Sigel and Barnard, 1984). Since this initial identification of subunits, cloning experiments have revealed a plethora of $GABA_A$ receptor subunits, encoded by separate genes, which combine to form $GABA_A$ receptors (see Sieghart, 1995 for review).

$GABA_A$ Receptor Structure and Function

The inhibitory actions of GABA were shown to result from an increase in neuronal permeability to chloride; this change in permeability could be antagonized by picrotoxin (Takeuchi and Takeuchi, 1969) and bicuculline (Nistri and Constanti, 1979). Electrophysiology studies in 1983 demonstrated that two GABA molecules must bind to cause a high probability of channel opening *in vivo* (Sakmann et al., 1983), and the Hill coefficient for GABA binding has been shown to be greater than one (Akaike et al., 1984). In adult rat hippocampal dentate gyrus cells, the average conductance of a single synaptic connection was 200-400pS and these cells demonstrated monoexponetial decay (Otis and Mody 1992). Furthermore, GABA-induced currents desensitized in a dose-dependent manner, as a result of a decrease in the probablity of channel openings (Weiss et al., 1988), and this $GABA_A$ receptor desensitizatiton was voltage-dependent (Hablitz, 1992).

It was not until a search was undertaken for the site of action of the benzodiazepines that the $GABA_A$ receptor was isolated and characterized (Sigel et al, 1983; Sigel and Barnard, 1984). The purified benzodiazepine receptors' characteristics were similar to the native environment when comparing benzodiazepine, GABA and muscimol binding. The subsequent identification of the subunit isoforms began with probing bovine brain cDNA

libraries with oligonucleotides. The two original cDNAs identified encoded two polypeptides, 456 and 474 amino acids in length, which were named α1 and β1 (Scholfield et al., 1987). One of the next to be identified was a 451 amino acid α2-subunit which had over 70% homology with the α1-subunit and less than 40% homology with the β1-subunit (Levitan et al., 1988). Based on the conserved sequences between subunit cDNAs previously described, the remaining isoforms have primarily been identified by screening brain cDNA libraries at low stringency with cDNA or degenerate oligonucleotide probes. Sequence homology of 70-80% defined a subunit class and 30-40% sequence homology separated these subunit classes. Over the next several years cDNAs encoding the remaining rat subunit isoforms were then identified and sequenced: α1 (Malherbe et al., 1990), α2 (Khrestchatisky et al., 1991), α3 (Majherbe et al., 1990), α4 (Wisden et al., 1991), α5 (Khrestchatisky et al., 1991; Pritcehtt and Seeburg 1990; Malherbe et al., 1990), α6 (Luddens et al., 1990), β1 (Ymer et al., 1989; Malherbe et al., 1990), β2 (Ymer et al., 1989; Lolait et al., 1989), β3 (Ymer et al., 1989; Lolait et al., 1989), γ1 (Ymer et al., 1990), γ2 (Shivers et al., 1989; Malherbe et al., 1990), γ3 (Knoflach et al., 1991; Herb et al., 1992), and δ (Shivers et al., 1989).

There is 20-30% sequence homology between the $GABA_A$ receptor and the nAch, Gly, and $5HT_3$ receptors, therefore the $GABA_A$ receptor is a member of the ligand-gated ion channel (LGIC) superfamily. The charactersitics of this superfamily include: (1) 4 putative transmembrane domains, (2) a long cytoplasmic loop between the third and fourth transmembrane domains, (3) a putative signal peptide, (4) 2 conserved cysteines in the extracellular N-terminal domain which presumably form a disulfide bridge, (5) N-glycosylation sites on the N-terminal domain and (6) putative phosphorylation sites in the long cytoplasmic loop. The regions of greatest homology are the putative transmembrane domains and the N-terminal domain; the regions of least homology are the putative signal peptide and the large cytoplasmic loop between the third and fourth transmembrane domains. Phosphorylation of this cytoplasmic loop may be a form of regulation, and *in*

vitro, both protein kinase A and C (PKA and PKC, respectively) have been shown to phosphorylate particular subunit isoforms (Browning et al., 1990; Sigel et al., 1991). *In vivo*, they have been also shown to modify $GABA_A$ receptor function (Kellenberger et al., 1992; Moss et al., 1992a,b). In $GABA_A$ receptor clones, many isoforms have consensus sequences for PKA, PKC and tyrosine kinase (Whiting et al., 1995).

The quaternary structure of the $GABA_A$ receptor is thought to be similar to that of the nAch receptor (Unwin 1995). The physiochemical properties of the solubilized receptor (Stephenson, 1988) and electron micrographic images of purified $GABA_A$ receptors suggest a pentameric structure (Nayeem et al., 1994). The stoichiometry of the $GABA_A$ receptor is still a matter of debate. α/β combinations have been shown to assemble most efficiently (Verdoon et al., 1990), but GABA recognition may not be dependent on a single subunit. More recently, the binding of GABA molecules and GABA-site agonists have been shown to be dependent on the α- and β-subunits (Sigel et al., 1992; Zezula et al., 1996; Westh-Hansen et al., 1997). Mutagenesis studies have illustrated that high and low affinity GABA binding may occur at the interface between α-β subunits (Sigel et al., 1992; Amin and Weiss, 1993; Sigel and Buhr, 1997; Westh-Hansen et al., 1997).

Similar to agonist binding sites on the nAch receptor, the benzodiazepine binding regions may be homologous to the GABA agonist binding sites, yet occur at different subunit interfaces (Galzi and Changeux, 1995). Benzodiazepine sensitivity is highly dependent on the presence of the α- and γ-subunits. The presence of the γ2-subunit in combination with an α subunit for benzodiazepine binding was initially illustrated (Pritchett et al., 1988) and when the γ-subunit was included in the combination with αxβx, the affinity for GABA declined (Sigel et al., 1990; Angelotti and MacDonald, 1993). Moreover, there was a dramatic increase in flunitrazepam binding in recombinant α1β1γ2 receptors compared to those combinations which did not include the γ-subunit (Moss et al., 1991). Mutagenesis and photolabeling studies indicate that the benzodiazepine recognition site may be determined by the α-γ subunit interface (Pritchett et al., 1989; Sigel et al.,

1990; Duncalfe et al., 1996; Amin et al., 1997; Sigel and Buhr, 1997; Wingrove et al., 1997).

The number of possible receptor subtypes, based on the different combinations of different isoforms, is greater than 500 000. However, with limitations of a fully functional receptor (Pritchett et al., 1989), and restrictions based on positioning, the number of $GABA_A$ receptor subtypes thought to exist is dramatically reduced. *In situ* hybridization mapping, immunoprecipitation, Western blotting, and immunochemical localization studies are being used to determine the subunit combination *in vivo*. A favored model involves 2xα, 1xβ, and 2xγ (Luddens et al., 1991; McKernan et al., 1991; Quirk et al., 1994b); yet, if there were 2xα and 2xγ subunit isoforms present there would be a possibility for two benzodiazepine binding sites. Nevertheless, more recent studies indicate the $GABA_A$ receptor pentameric model may involve 2xα, 2xβ, and 1xγ (Chang et al., 1996; Tretter et al., 1997); however, the δ and ε subunit isoforms may replace the γ subunit within the receptor complex (Quirk et al., 1994a; Davies et al., 1997; Whiting et al., 1997).

The latest estimate for the number of $GABA_A$ receptor subtypes is greater than 10, the predominante subtypes being α1βxγ2 in the cortex and α1βxγ2 or α6βxγ2 in the cerebellum (Fritschy and Mohler, 1995; Whiting et al., 1995; Bohlhalter et al., 1996; Jechlinger et al., 1998). However with the evolution of better techniques in recent years, the estimate of $GABA_A$ receptor subtype numbers will experience a certain degree of adjustment. It has been shown previously that the subunit composition, and hence the receptor subtype, determines the functional and pharmacological profile of the $GABA_A$ receptor (Pritchett et al., 1989; Levitan et al., 1988; Wafford et al., 1990; Puia et al., 1992; Hadingham et al., 1996; Wafford et al., 1996; Whittemore et al., 1996).

The α-subunit

The importance of the α-subunit isoform, not only for benzodiazepine binding, but also for receptor function, was recently demonstrated when the inclusion of the α subunit

into the $GABA_A$ receptor was an essential prerequisite not only for the expression of other subunit classes, but also for complete receptor formation *in vivo* (Jones et al., 1997; Fritschy et al., 1997). The first divison of receptor subtypes was based on pharmacological observation, and two benzodiazepine subtypes of $GABA_A$ receptors were reported. With molecular cloning this divergence was found to result from different α-subunits. The benzodiazepine type 1 (BZ1) and benzodiazepine type 2 (BZ2) pharmacologies were elucidated using HEK 293 cells transiently expressing receptors of various α-subunit isoforms in combination with β1γ2 subunits (Pritchett et al., 1989). This division of subtypes was based on the affinity of subunit isoform combinations for two compounds: CL 218872 and β-CCM. The α1-subunit containing receptor had a high affinity for these agents whereas the α2/α3-subunit containing combinations had a lower affinity. Further separation into subtypes occurred when α5βxγ2 receptors showed similar affinity as the α2/α3-subunit containing receptors for CL 218872 and β-CCM, but dramatically less affinity for the compound zolpidem (Pritchett and Seeburg, 1990). The differences in the affinity for zolpidem between α-subunit containing receptors demonstrated distinct subtypes based on pharmacology for [^{3}H]-benzodiazepine displacement: α1-subunit containing receptors have a 100-fold higher affinity for zolpidem than α2/α3-subunit containing receptors and a 1000-fold higher affinity than α5-subunit containing receptors (McKernan et al., 1991; Hadingham et al., 1993a). These α5-subunit containing receptors have a 10-15 fold higher affinity for Ro 15-4513 than the α1-, α2- or α3-subunit containing receptors (Hadingham et al., 1996). For the purpose of this thesis, the α5-subunit containing receptor subtype was named benzodiazepine type 3 (BZ3).

Subtle differences within and between these subtypes were also distinguished with respect to pharmacology. For instance, the GABA-induced shift in benzodiazepine binding was greater in α3-subunit containing receptors than in α1-subunit containing receptors, which was greater than α5-subunit containing receptors. (Faure-Halley et al., 1993; Wafford et al., 1993a; Graham et al., 1996). However, the rank-order affinity for

diazepam was greater in α5β2γ2 receptors than α3- and α1-subunit containing receptors, (Graham et al., 1996). The affinity for GABA between the α-subunit variant receptors varies as well. The α3-subunit receptors have a lower GABA affinity than α1- or α2-subunit receptors (Ebert et al., 1994; White et al., 1995), and α5-subunit receptors have the highest GABA affinity (Ebert et al., 1994), with a 70-fold difference between the α3- and α5-subunit containing receptors. The affinity of GABA, however, was unaltered in the presence of two different α-subunit isoforms in combination (Ebert et al., 1994).

Further division of benzodiazepine subtypes was made when the combination of α4- or α6-subunits were combined with βxγ2 subunits. These receptors showed a high affinity for Ro 15-4513, moderate affinity for the classical antagonist Ro 15-1788 (flumazenil) and no classical benzodiazepine affinity (diazepam, flunitrazepam, midazolam) (Luddens et al., 1990). These receptor subtypes became known as diazepam insensitive (DIS). The α6-containing receptors are primarily localized to cerebellar granule cells (Wisden et al., 1991; Hadingham et al., 1996) and that α4-subunit mRNA is of very low abundance but relatively diffusely distributed throughout the CNS (Wisden et al., 1991; Benke et al., 1997; Wafford et al., 1996). The loss of diazepam binding appears to be conferred by the N-terminal region of the subunit, where the histidine 101 in the α1-subunit isoform is replaced by an arginine in the α6-subunit; mutation of this amino acid has confirmed its involvement in diazepam sensitivity (Fisher et al., 1997). Increased sensitivity to GABA by α6-subunit containing receptors relative to α1-subunit containing receptors has been demonstrated to be a contribution of both the amino- and carboxyl-terminals (Fisher et al., 1997), yet the N-terminal is primarily responsible for benzodiazepine binding. Although receptors which contain the α4- and α6-subunits are very similar in the degree of potentiation by GABA (Knoflach et al., 1996), and they lack classical benzodiazepine, CL 218872, zolpidem, and imidazenil binding (Benke et al., 1997; Hadingham et al., 1996; Whittemore et al., 1996; Wafford et al., 1996), there are distinct similarities and differences in certain pharmacological responses. In particular, the

classical partial agonist bretazenil potentiates GABA in a manner similar to full agonists at both α4- and α6-subunit containing receptors (Benke et al., 1997; Knoflach et al., 1996; Whittemore et al., 1996; Hadingham et al., 1996; Wafford et al., 1996); however, the maximal potentiation was greater for the α4-subunit containing than the α6-subunit containing receptor (Knoflach et al, 1996). Further these recombinant studies have demonstrated that α4- and α6-subunit containing receptors have altered pharmacodynamic responses. For instance the benzodiazepine inverse agonist, Ro 15-4513, generally demonstrates negative modulatory actions, but this converts to a positive modulatory role with the α4- and α6-subunit containing receptors (Hadingham et al., 1996; Knoflach et al., 1996; Wafford et al., 1996; Whittemore et al., 1996). However, unlike the α4-subunit subtype which has been shown to display reduced partial GABA-site agonist (P4S and THIP) potentiation relative to the α6-subunit containing $GABA_A$ receptors (Wafford et al., 1996), the α6-subunit containing receptor can still be directly activated by pentobarbital (Whittemore et al., 1996; Hadingham et al., 1996). Furthermore, furosemide has been shown to be a selective α6-subunit containing receptor antagonist (Wafford et al, 1996). For the purpose of this study, the classification of DIS binding includes both the α4- and α6-subunit $GABA_A$ receptor subtypes in the cerebellum; however, because of the distinct localization of α6-subunit expression, cortical and hippocampal DIS binding was primarily assumed to be a result of the α4-subunit containing receptors. However, the ability to predict pharmacological properties of the α-subunit containing receptors cannot be performed with different members of the β-subunit class, and likewise, the pharmacological profile of the βsubunits cannot be accurately determined or compared using different α subunits in recombinant receptors (White et al., 1996).

The β-subunit

The importance of the β subunit in functional $GABA_A$ receptor formation has been demonstrated with regard to cellular localization and clustering (Angelotti et al., 1993;

Connolly et al., 1996a,b) . The β subunit has been primarily implicated in the binding of GABA at the $GABA_A$ receptor, and comparisons between the different isoforms with a number of benzodiazepines demonstrated no significantly altered benzodiazepine binding affinity or efficacy (Hadingham et al., 1993b). Immunoprecipitation studies illustrated that 55-60% of $GABA_A$ receptors contain the β2 subunit whereas 19-25% and 16-18% contain the β3- and β1-subunit isoforms, respectively (Benke et al., 1994). Further, the β2 subunit appeared to preferentially associate with α1 and γ2- subunits, and the β1- and β3- isoforms with α3 and α5 subunits (Benke et al., 1994). When in combination with α1γx, there were no alterations in efficacy or affinity amongst the β-subunit isoforms (Ebert et al., 1994), and the GABA potentiation was greater with diazepam than bretazenil at all α1βxγ2 subunit combinations (Puia et al., 1992). However, when α5 replaced α1, the affinity for $GABA_A$ receptor benzodiazepine-site agonists were ranked β3>β2>β1 (Ebert et al., 1994). This small degree of difference in benzodiazepine pharmacology manipulation was further shown with zolpidem affinity, where β2-subunit containing receptors had a 5-8 fold higher affinity than the β1- or β3-subunit containing receptors. This indicated that β2-subunit containing receptors displayed BZ1 type pharmacology and β1-, β3-subunit receptors demonstrated different pharmacologies (Benke et al., 1994). The kinetics of the $GABA_A$ receptor activation may also be affected by the β-subunit isoform: when in combination with the α5γ2L, the β2-subunit containing receptor desensitized faster than β1- and β3-subunit containing receptor subtypes (Burgard et al., 1996). Further, the affinity for GABA appears to be somewhat βsubunit dependent according to a variety of reports (Pritchett et al., 1989; Hadingham et al., 1993b; Ebert et al., 1994; Burgard et al., 1996). However a clear rank-order pattern has not been demonstrated. A unique characteristic of the β-subunit was recently demonstrated when the anticonvulsant loreclezole was found to bind to the $GABA_A$ receptor with 300-fold greater affinity when the β1-isoform was present as compared to the β2- or β3-subunits (Hadingham et al., 1993b). In summary, it

appears certain combinations containing the β-subunit isoforms can influence benzodiazepine pharmacology and $GABA_A$ receptor activation.

The γ-subunit

In addition to the α subunit, the γ subunit plays an important role in benzodiazepine site binding and modulation of the $GABA_A$ receptor. The expression of the γ2-subunit mRNA is ubiquitous; however, the expression of γ3-subunit isoform gene expression is low relative to γ1- and γ2-subunit gene expression (Herb et al., 1992; Wafford et al., 1993b). The first examination of this subunit family in recombinant systems demonstrated that the presence of the γ2-subunit isoform allowed significant modulation by benzodiazepine ligands (Pritchett et al., 1988). Examination of recombinant expression systems has since demonstrated that replacing this isoform with either γ1 or γ3 subunit alters benzodiazepine modulation and affinity at the $GABA_A$ receptor.

When γ2subunit was replaced by either γ1 or γ3 subunit in the α1, α2, α3, or α5 subunit and βx subunit combinations, the efficacy for diazepam was similar to that of bretazenil (Puia et al., 1992). The replacement of the γ2-subunit isoform with the γ1-subunit isoform in recombinantly expressed receptors demonstrated a overall reduced ability for benzodiazepine agonist-induced GABA potentiation (Wafford et al., 1993b). Generally, the BZ1 selective compounds had a lower maximal potentiation and the CL 218872 pharmacology switched from partial agonism to a full positive modulation at γ1-subunit containing receptors; moreover, zolpidem modulation was more selective for the γ1-subunit receptor subtypes. Further, the switch from γ2- to γ1-subunit isoform containing receptor changed the inverse agonists DMCM and Ro 15-4513 into positive modulators of GABA-gated chloride current (Puia et al., 1992; Wafford et al., 1993b).

The formation of $GABA_A$ receptors which contain γ3-subunit isoforms resulted in receptors which demonstrated lower affinity for diazepam and no affinity for zolpidem (Graham et al., 1996). The ability of benzodiazepine ligands to potentiate GABA enhanced

chloride current at $GABA_A$ receptor subtypes containing the γ3-subunit was compromised and the affinities of benzodiazepine agonists for the receptor were dramatically reduced (Knoflach et al., 1991; Herb et al., 1992). The affinities for antagonists and inverse agonists were similar between the γ2- and γ3-subunit expressing receptor subtypes (Herb et al., 1992); however, DMCM demonstrated a more pronounced depression of GABA activated chloride current (Knoflach et al., 1991). The relative importance of subunit isoform subtype pharmacologies demonstrates that the definition of these $GABA_A$ receptor subtypes based on pharmacology of the α-subunit isoform expression is limiting. In an attempt to define benzodiazepine receptor subtypes with regard to pharmacology, re-classification is required in order to account for the alterations in receptor modulation which occur as a result of a single β/γ isoform switch.

Other subunits

In this study, the regulation of only the $GABA_A$ receptor α-, β-, and γ-subunit isoforms was examined. These subunits are generally widely distributed in the CNS and their expression correlates well with expected benzodiazepine pharmacology. However, additional subunits have been identified, with limited anatomical distribution and poorly described pharmacological properties. The recently cloned π subunit has predominantly been found in the reproductive tract and, when co-expressed with α1βxγ2, a reduced sensitivity to the steroid pregnanalone was exhibited (Hedbloom and Kirkness, 1997). Three other subunit families have been identified, but their anatomical localization within the brain is limited. The cloned δ subunit (Shivers et al., 1989) is expressed primarily in the thalamus and the cerebellum (Wisden et al., 1992) and the replacement of the γ-subunit by the δ subunit in recombinant receptors resulted in benzodiazepine insensitivity (Shivers et al., 1989; Quirk et al., 1994a). The recent discovery of the ε subunit, which has a distribution limited to the amygdala and subthalamic nuclei in the CNS, demonstrated that replacement of the γ-subunit with the ε subunit revealed $GABA_A$ receptors which were

insensitive to barbiturates and anesthetics (Davies et al., 1997), and may be insensitive to benzodiazepines (Davies et al., 1997; Whiting et al., 1997). The ρ-subunit family is primarily expressed in the retina and forms the unique $GABA_C$ receptor which does not display benzodiazepine, barbiturate, or $GABA_A$ receptor antagonist bicuculline modulation (Cutting et al., 1991; Ogurusu and Shingai, 1996).

Developmental Subunit Expression

A number of studies have examined the embryonic and postnatal development of the $GABA_A$ receptor and the expression of subunit isoforms. Using immunoreactivity, Fritschy's group (1994) showed very low and restricted expression of the α1-subunit gene in rat embryos; however, within the first week following birth, there was a dramatic increase in both expression level and distribution. This dramatic change in α1-subunit gene expression preceded a decrease in α2-subunit gene expression, which during embryonic development was highly expressed and widely distributed. Yet, postnatally there was a certain degree of coexistence between the expression of α1 and α2 subunits. This pattern of change was different from the expression of the β2/β3-subunit genes, which were ubiquitous during both development and in adulthood. In the neonate a number of subunit isoforms have been reported to be expressed at high levels: α2, α3, α5, β3, and γ2 (Fuchs and Sieghart, 1989; Laurie et al., 1992; Poulter et al., 1992; 1993). Prenatally, the $GABA_A$ receptors demonstrate predominantly BZ2 subtype binding characteristics (Lippa et al., 1981). Moreover, at early developmental stages there was an increased responsiveness to GABA and activation of $GABA_A$ receptors was linked to neuronal excitation and increased Ca^{++} influx (Cherubini et al., 1991). This shift to $GABA_A$ receptor activation, leading to neuronal inhibition, follows the developmental shift in the membrane chloride gradient. This replacement/switching of specific subunits during development (i.e. the α2-subunit expression switch to α1-subunit expression) corresponds to, or just precedes the period of synaptogensis (Poulter et al., 1992; 1993; Fritschy et al., 1994).

Benzodiazepine Pharmacology

The search for compounds which were more selective than the classical benzodiazepines lead to the development of agents with different pharmacodynamic profiles. The range of compounds included classical benzodiazepines (diazepam) which have positive intrinsic efficacy and enhance GABA-gated chloride influx. Their pharmacological profile includes anxiolysis, sedation, muscle relaxation and anticonvulsant effects over a range of receptor occupancy (Braestrup and Neilsen, 1983; Haefely et al., 1985). At the opposite end of the spectrum are the inverse agonists (DMCM) which cause a decrease in chloride influx (MacDonald and Twyman, 1992) and are defined as having negative intrinsic efficacy; these compounds are convulsants, stimulants and anxiogenics (Braestrup et al., 1983). Between the extremes, however, a range of compounds exist which have partial agonist, partial inverse agonist and antagonist activity. The specific efficacy or pharmacodynamic profile of a specific ligand has, however, been demonstrated to be dependent on the specific subunit composition of the $GABA_A$ receptor subtype (Puia et al., 1992; Wafford et al., 1993b; Hadingham et al., 1996; Knoflach et al., 1996; Wafford et al., 1996; Whittemore et al., 1996) The classical $GABA_A$ receptor antagonist (Ro 15-1788/flumazenil) has very weak or no intrinsic activity, and therefore, does not alter GABAergic transmission, yet may inhibit the the actions of other benzodiazepines (Braestrup et al., 1983). Historically, partial agonists have weaker intrinsic efficacy than the full agonists, and the magnitude of GABA-gated chloride current measured *in vitro* is dependent upon the individual intrinsic activity or efficacy of the partial agonist (Maksay, 1993). In general, the partial agonist pharmacological profile only includes anxiolytic and anticonvulsant effects in animals (Pieri et al., 1988; Potier et al., 1988; Belzung et al., 1989; Haefely et al., 1990; Facklam et al., 1992a,b; Haefely et al., 1992; Martin et al., 1993; Doble et al., 1993; Guisti et al., 1993) even at receptor saturation. These effects are generated at lower fractional occupancies than the sedative and myorelaxant effects

(Facklam et al., 1992a,b), yet the partial agonists have been shown to antagonize the sedative and muscle relaxant effects of the full agonists (Haefely et al., 1990). Further, because of the lower intrinsic efficacy, receptor occupation by partial agonists does not lead to over-stimulation of the $GABA_A$ receptor, and therefore chronic exposure would not cause receptor desensitization (Haefely et al., 1990). This lack of desensitization may be an important factor with respect to the partial agonists' decreased ability to induce specific $GABA_A$ receptor alterations and tolerance after extended treatment.

The partial agonist bretazenil (Ro 16-6028) was developed as a novel agent which has intermediary intrinsic activity (Guisti et al., 1993; Martin et al., 1993) yet is very potent at the $GABA_A$ receptor. In fact, it has a 10-20X higher affinity for the $GABA_A$ receptor than diazepam in mice (Haefely et al, 1991; Giusti et al., 1993), is more potent in rat (Martin et al., 1988) and can dose-dependently antagonize the effects of diazepam to levels which are similar to bretazenil alone (Haefely et al, 1992; Puia et al., 1992; Finn and Gee, 1993). However, like all partial agonists, bretazenil requires higher receptor occupancy than diazepam for the same magnitude of effects (Potier et al., 1988).

Chronic exposure to bretazenil and other partial agonists (Doble et al., 1993; Impagnatiello et al., 1996) does not result in the development of anticonvulsant (Haigh and Feely, 1988; Hernandez et al., 1989; Rundfelt et al., 1995) and anxiolytic (Miller et al, 1990) tolerance and withdrawal syndrome (Moreau et al., 1990), which is an advantage over the full agonist benzodiazepines. However, in one report, high doses of chronic bretazenil were found to produce tolerance to other benzodiazepine and benzodiazepine-like compounds in the rat (Bronson 1995). In man, reports of bretazenil partial agonist activity vary; initial studies in man reported that anxiolytic doses were not associated with sedation (Haefely et al., 1990), but this was in disagreement with subjective tests of sedation which indicated that high doses of bretazenil produce the full range of pharmacological effects (Merz, 1984). It has been reported again recently that with subjective measures of sedation there was an association with sedative and anxiolytic effects during bretazenil treatment

(Van Seveninck et al., 1996). Nonetheless, the majority of reports in animals and *in vitro* models indicates the possibility that partial agonist chronic treatment may avoid the development of tolerance and, hence, dependence.

Benzodiazepine Depedence: tolerance and withdrawal

Drug dependence has been defined in a number of ways; generally the dependence syndrome is thought of as drug seeking behavior, where drug use is given a priority over all other behaviors (Jaffe, 1990). Accordingly, dependence and tolerance are separate factors, although the development of tolerance may contribute to the development of dependence. However, benzodiazepine tolerance and withdrawal may be viewed as manifestations of benzodiazepine dependence (Lader and File, 1987). In this instance the dependence syndrome may be defined to include drug-seeking behavior, tolerance development, and marked withdrawal effects upon drug cessation. Dependence results from an altered physiological state caused by changes or adaptations in the physiological system in response to drug administration. The development of behavioral tolerance following repeated administration results in either a reduced reponse to a given dose of drug, or the need to increase the dosage to maintain the same original drug effects (Jaffe, 1990; Nestler, 1992). There are three processes of tolerance development: dispositional tolerance (pharmacokinetic mechansims), learned tolerance (conditioned), and functional tolerance (pharmacodynmic mechanisms) (Ritzman et al., 1984; Loshcer et al., 1996a,b; Rundfeldt et al., 1996). Dispositional tolerance has been demonstrated to not be a factor in benzodiazepine tolerance (Greenblatt and Shader, 1986; File 1982a,b; Lister 1983), and associated learning processes may primarily contribute to the retention of tolerance (File 1982b; Loscher et al., 1996a,b). There are two distinct models of functional tolerance, decremental and oppositional, whereby adaptive changes are recruited in an attempt to

maintain homeostatic balance (Littleton and Harper, 1990). The withdrawal syndrome is believed to be a result of dependence, and in the instance of the benzodiazepine adminsitration may be thought of as a consequence of oppostional tolerance (Lader and File, 1987; File and Fernandes, 1994; Fernandes et al., 1996). Furthermore tolerance and withdrawal syndromes are not continuous across the behavioral spectrum; there are different rates of tolerance development to different behaviors, and the development of one manifestation of dependence is not always associated with the expression of the other. (File 1985; Lader and File, 1987; File and Fernandes, 1994). Therefore it has not been established if the development of benzodiazepine tolerance and the incidence of withdrawal are both a result of the same underlying devices. More importantly, it is unknown whether the underlying mechanisms for the different behavioral alterations in tolerance development are the same underlying neurochemical adaptation or not. In the context of this thesis, dependence refers to chronic benzodiazepine induced alterations; therefore, tolerance and withdrawal are a manifestation of this syndrome.

Specific Aims

The aim of the work described in this dissertation was to gain a greater understanding of the mechanisms underlying the neurochemical and behavioral adaptations which occur following chronic benzodiazepine exposure. The primary hypothesis is that chronic full agonist (diazepam) exposure, which induces tolerance, results in concomitant up- and down-regulation of subunit isoform gene expression, which consequently alters the profile of $GABA_A$ receptor subtypes. These changes in $GABA_A$ receptor steady-state mRNA levels result from exposure to diazepam and underlie the changes in $GABA_A$ receptor protein expression, subsequently altering the benzodiazepine recognition properties and thereby resulting in the development of tolerance.

Initially, the first objective was to establish a method for continuous delivery of diazepam over an extended period. To accomplish this, an appropriate solvent was found which maintains diazepam in solution and does not compromise long term delivery from osmotic minipumps (Chapter 2). The second aim was to examine the effects of continuous diazepam treatment length and withdrawal on $GABA_A$ receptor steady-state mRNA levels (Chapter 3). Cortical, cerebellar, and hippocampal changes in 12 $GABA_A$ receptor subunits were examined after 7, 14, or 28 days treatment and then after 28 days treatment and 3 days drug cessation. The third objective specifically utilized 14 days chronic diazepam exposure, investigating the parameter of dosing paradigm. Following equivalent daily diazepam doses administered via two dosing regimes that differed in their kinetics of drug delivery and, therefore receptor occupancy, $GABA_A$ receptor gene expression and benzodiazepine binding capacity were examined in the cortex (Chapter 4). Further analysis of these two dosing paradigms were investigated with regard to their relationship with the temporal development of behavioral and anticonvulsant tolerance (Chapter 5). This fourth specific aim was to monitor changes in animal behavior following chronic diazepam exposure, and if established, to ask whether these alterations could be related to the

treatment specific changes in the neurochemistry of the GABAergic system. The final goal was to examine the steady-state mRNA levels after 14 days daily injection with the full agonist diazepam and the partial agonists bretazenil and RP 60503, where chronic exposure to partial agonists does not result in the development of tolerance. Three brain regions (cortex, cerebellum and hippocampus) were examined for differential patterns of change in $GABA_A$ receptor gene expression (Chapter 6). Further, examination of protein expression by analysis of the benzodiazepine binding profile was employed to establish if changes in the recognition properties of $GABA_A$ receptors correlate with alterations in $GABA_A$ receptor subunit gene expression following chronic treatment.

[1]CHAPTER 2

Dimethyl Sulfoxide/Propylene Glycol is a Suitable Solvent for the Delivery of Diazepam from Osmotic Minipumps

[1] A version of this chapter has been published. Arnot M.I., Bateson A.N. and Martin I.L. (1995) *J. Pharmacol. Toxicol. Meths.* **36**(1): 29-31.

Introduction

Alzet® osmotic minipumps were introduced in 1983 for the continuous long term delivery of drugs in experimental studies. Their operation is dependent on the osmotic pressure developed between a semipermeable capsule and an impermeable pouch which contains a drug solution of known concentration. The pressure extrudes the solution from this pouch at a rate that allows relatively constant drug delivery over periods of up to 28 days. These pumps have proved extremely useful for the delivery of drugs which are water soluble (e.g. Löscher, 1986).

Alzet® osmotic minipumps are particularly attractive for the long-term exposure of experimental animals to benzodiazepines; however, many of these drugs are poorly soluble in aqueous solvents and several alternative solvents have been investigated (Hawkins et al., 1988; Marley et al., 1991; Miller et al., 1988; Primus and Gallager, 1992; Torchin et al., 1993). While 100% dimethyl sulfoxide is an appropriate solvent for diazepam, it has been found to cause significant failure of these pumps (personal communication, ALZA Corporation). On the other hand, aqueous solutions of several glycol analogues fail to retain the drugs in solution, although 100% tetraglycol appears not to suffer from this disadvantage. The work described here provides a detailed comparison between 50% dimethyl sulfoxide/50% propylene glycol (v/v) and 100% tetraglycol solvents for drug delivery, and has been conducted *in vitro* using both radioligand binding assays and [^{3}H]-labeled tracers. It appears that diazepam dissolved in 50% dimethyl sulfoxide/50% propylene glycol provides (v/v) appropriate drug delivery.

Material and Methods

Osmotic minipumps (model 2ML4, 28 day, lot #044301) were obtained from ALZA Corporation, Palo Alto, California. Diazepam and clonazepam were generous gifts from Hoffmann-La Roche, Basel, Switzerland; [^{3}H]-Ro 15-1788 (specific activity of 75.3 Ci/mmol) and [^{3}H]-flunitrazepam (specific activity 82 Ci/mmol) were obtained from NEN-Dupont, Canada and [^{3}H]-water (specific activity 100 mCi/ml) was purchased from ICN. Dimethyl sulfoxide (DMSO) and tetraglycol were obtained from Sigma, and propylene glycol U.S.D. (PG) was obtained from the University of Alberta Hospital Pharmacy. Saline solutions were 0.9% (w/v).

Diazepam was dissolved as a stock solution of 50% DMSO/50% PG (v/v) at a concentration of 1.5 mg/ml, and three pumps were filled with this solution according to the IBM Pump Fill Protocol (Greenshaw, 1986). After priming overnight submersed in a vial of 10 ml saline at room temperature, pumps were transferred to beakers containing 70 ml saline; the beakers were covered with parafilm and placed in a 37°C incubator for the duration of the study. To ensure that the DMSO/PG solvent combination allowed diazepam release, aliquots were removed from the solution in the beaker and the pumps transferred to new beakers containing 70 ml of saline, covered and again placed at 37°C. Samples were taken for the determination of diazepam concentration at day 2 and 7. Drug concentrations were determined by competitive displacement of the specific binding of 0.5 nM [^{3}H]-flunitrazepam in rat cortex membranes (Owen et al., 1979). Non-specific binding was defined with 3 µM clonazepam. Standard curves were constructed with 2-1024 nM diazepam (10 mM stock in 95% v/v ethanol) diluted with 50 mM Tris-HCl (pH 7). Aliquots taken from the beaker which contained the pumps were also diluted with 50 mM Tris-HCl (pH 7) in order to allow an equal volume to be added to each reaction tube. Each unknown was determined in triplicate in two separate experiments.

In order to explore the effects of different vehicles on the delivery rate, three pumps were filled with diazepam at a concentration of 1 mg/ml in either 50% DMSO/50% PG (v/v) or 100% tetraglycol and were supplemented with 3 μCi [^{3}H]-Ro 15-1788 per 10 ml solvent. A further series of pumps containing saline and water were supplemented with 30 μCi [^{3}H]-water per 10 ml vehicle to examine: (1) the stability of the pump over 28 days, (2) whether pump integrity was maintained in aqueous solvents, and (3) if [^{3}H]-Ro 15-1788 was binding to the pump lining. The delivery of pump contents into saline was monitored as described above. The radioactivity was determined by liquid scintillation counting of 100 μl samples in triplicate using CytoScint (ICN). Statistical analysis used Student's non-paired *t*-test, one-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA).

Results

Initial studies to quantitate the amount of diazepam discharged from the minipumps, containing this drug as a solution in 50% DMSO/50% PG (v/v), indicated that there was a significant decline from day 2 to day 7 ($P<0.03$, n=3; Figure 2.1). Using the same experimental conditions, but supplementing the diazepam solution within the osmotic minipump with radiolabeled benzodiazepine tracer ([^{3}H]-Ro 15-1788), a significant decline was also noted over a period of 28 days, the final release rate decreasing by 30.4 ± 2,3% from day 7 to day 28 ($P<0.01$, n=3; Figure 2.2). The alternate solvent, tetraglycol, resulted in a much larger variation in the release of the drug over the same period of time, which varied from 170.7 ± 17.3% on day 7 to 60.5 ± 6.6% of the theoretical rate at day 28 ($P<0.01$, n=3; Figure 2.2). Further, there was a significant variation ($P<0.01$) between the three individual pumps with this solvent at early time points, which was not observed with the DMSO/PG solvent. A time dependent decrease was also found in the experiments

in which saline, supplemented with [^{3}H]-Ro 15-1788, replaced the diazepam solution within the pump (Figure 2.2) such that the discharge rate at day 28 was only 44.4 ± 1.0 % of that at day 7 ($P< 0.01$, n=3).

In the saline-filled pumps, when [^{3}H]-Ro 15-1788 tracer was replaced by [^{3}H]-water the output rate on day 28 was 80.0 ± 2.2% of the rate on day 7 ($P<0.05$, n=3; Figure 2.3). Further, a decrease in release rate over time was also demonstrated when the pumps were filled with water supplemented with [^{3}H]-water: the output rate at day 28 was 75.4±12.5% of the output rate observed at day 7 ($P< 0.01$, n=3; Figure 3).

Discussion

The specifications of the Alzet® osmotic minipumps state that an output rate of 2.5± 0.5 µl/hr can be expected. However in each series of experiments reported here it was clear that a significant decline in the discharge rate occurs over the period of 28 days.

In the experiments in which the pumps were filled with water and the discharge rate monitored by supplementation of the internal pump solution with [^{3}H]-water, the rate fell by 24.6% between days 7 and 28. Pumps filled with saline and supplemented with [^{3}H]-Ro 15-1788 showed a marked decline in the discharge rate over the same period, but the magnitude of this decline was markedly reduced when the tracer was replaced with [^{3}H]-water (20% versus 56% respectively). This suggested that some of the benzodiazepine tracer may have suffered adsorption to the pump lining.

The primary purpose of this study was to ensure that diazepam, which is poorly soluble in aqueous solutions, could effectively be administered using osmotic minipumps. Using an *in vitro* model system, it proves possible to dissolve sufficient diazepam in 2 ml of a 1:1 mixture of DMSO and PG, which *in vivo* would allow continuous exposure of an animal to 15 mg/kg/day of this drug for a period of 28 days. Under the experimental

conditions using the [^{3}H]-labeled tracer, a decline in the discharge rate of the drug was found over this period, the average being 1.45% per day; measurement of diazepam using a radiolabeled displacement assay over a more limited period of time, indicates that the pump release rate is within these limits. However, this was not significantly different from that found when the pumps were filled with aqueous solutions, where the average rate was decreased 1.12% per day (the output rates have been normalized to 100% on the first measurement day to allow rational comparison). It was clear that the alternate solvent tetraglycol, which has been used previously for similar experiments (Torchin et al., 1993), produced a greater variation in the output rate, the minimum rate being 35% of that of the maximum rate, and a greater variation within the experimental period. This increased variation may be due to fluctuations in individual pump integrity where the solvent may have caused clogging of the pump head by degeneration of the reservoir or perhaps in some instances the degraded pump lining allowed leakage of the drug and tracer via the pump walls. (Personal observation based on pump changing to opaque and material found at bottom of beaker during time course.)

The experiments reported here suggest that diazepam may be effectively delivered chronically to experimental animals by the use of Alzet osmotic minipumps. The rate of delivery appears to decline over time with all vehicles tested. This chapter demonstrates that DMSO/PG delivers at a rate which is not significantly different from aqueous solvents but is able to retain diazepam in solution.

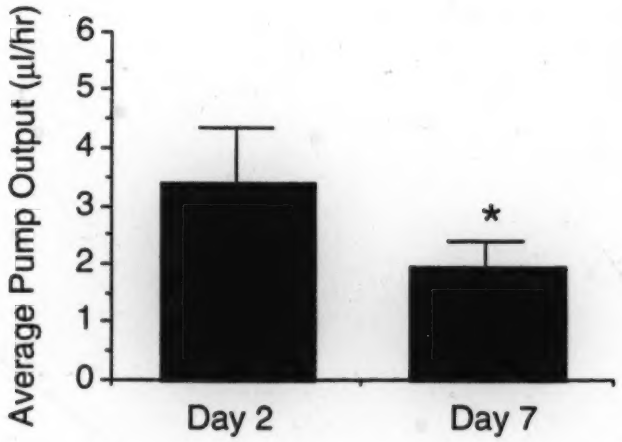


Figure 2.1:

Average pump output rate (µl/hr) of diazepam dissolved in the vehicle 50% DMSO/50% PG (v/v). Diazepam was quantified by competitive displacement of [^{3}H]-flunitrazepam from rat cortical membranes. Each bar represents the mean value from 3 pumps ± SEM. The asterisk denotes a significant difference between day 2 and day 7 ($P<0.03$, Student's non-paired *t*-test).

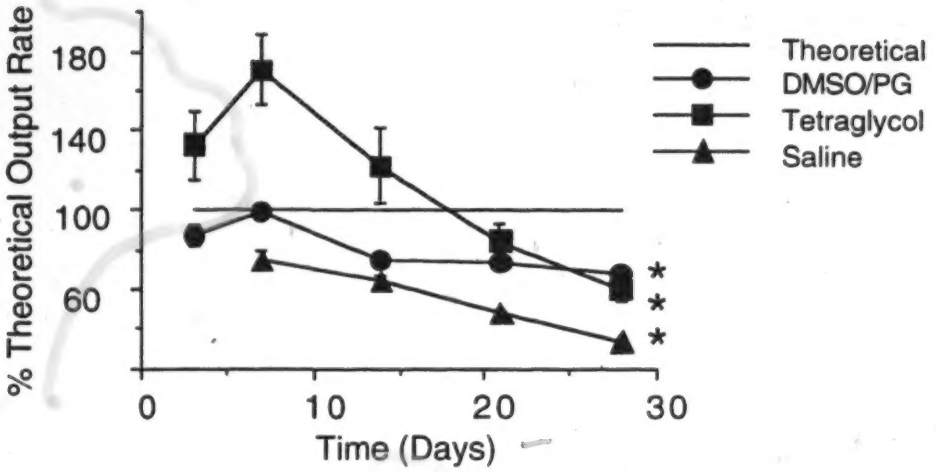


Figure 2.2:

Comparison of pump output rate using [^{3}H]-Ro 15-1788 tracer. All pumps contained diazepam except the saline-filled pump. Each point is the mean value from 3 pumps ± SEM normalized to the theoretical output rate. For certain data points the error bars are within the symbols. The theoretical output rate (5229 dpm/ml/day) was calculated on the basis of pump lot specification rate of 2.29 µl/hr with each pump containing 0.3 µCi [^{3}H]-Ro 15-1788 per ml solvent. Asterisks denote significant differences between output rates at day 7 and day 28 for each vehicle ($P<0.01$, one-way ANOVA)

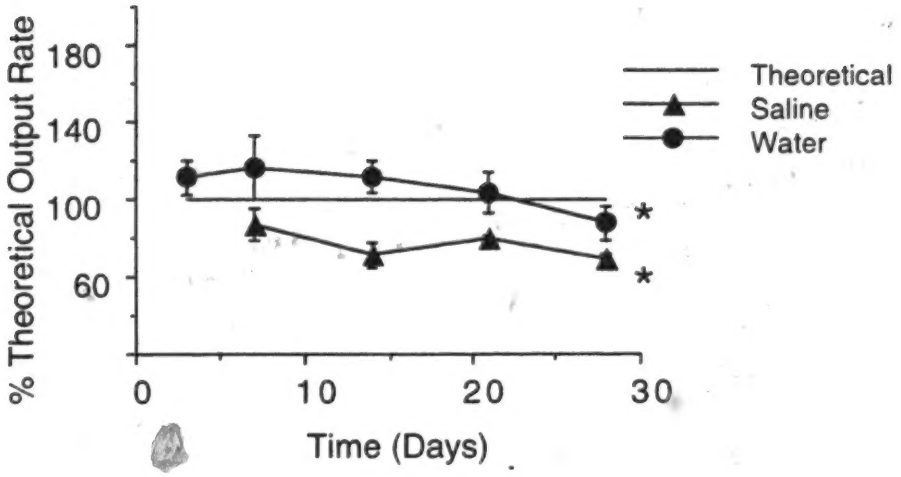


Figure 2.3:

Comparison of output rate when vehicles contained [^{3}H]-water tracer, but no diazepam. Each point is the mean value from 3 pumps ± SEM normalized to the theoretical output rate. The theoretical output rate (52290 dpm/ml/day) was calculated on the basis of the pump lot specification rate of 2.29 µl/hr with each pump containing 30 µCi [^{3}H]-water per ml vehicle. Asterisks denote significant differences between the output rates at day 7 and day 28 for each vehicle ($P<0.05$ for saline, and $P<0.01$ for water, one-way ANOVA).

CHAPTER 3

$GABA_A$ Receptor Isoform Gene Expression: Time Course Effects of Chronic Diazepam Exposure via Continuous Infusion

Introduction

Diazepam is a 1,4-benzodiazepine compound with sedative, anxiolytic, muscle relaxant and anticonvulsant properties. Therapeutic applications of diazepam are limited because there is evidence for dependence potential after chronic treatment (Tyrer, 1987; Roy-Byrne, 1991). Benzodiazepine dependence can be characterized by the development of tolerance to particular aspects of its pharmacological profile followed by a marked withdrawal syndrome on discontinuation (Chapter 1; Gordon, 1967; Clare 1971). The molecular mechanisms which underlie this phenomenon are poorly understood. Diazepam allosterically modulates $GABA_A$ receptors in the mammalian CNS, and the pharmacological characteristics of a given receptor subtype are dependent upon the specific isoform composition (see Chapter 1).

Chronic treatment with diazepam has been shown previously to alter some $GABA_A$ receptor steady-state mRNA levels (Heninger et al., 1990; Primus and Gallager, 1992; Wu et al., 1994b; Brett and Pratt, 1995; Holt et al., 1996; Impagnatiello et al., 1996), and these alterations may lead to the development of tolerance and withdrawal. However, the temporal development of these $GABA_A$ receptor mRNA steady-state level changes have not been explicitly examined. Further, in the previous studies, the method of drug treatment has not always been the same and alterations in $GABA_A$ receptor mRNA levels may be regime specific (see Chapter 4). The purpose of this study was to examine the time course of mRNA alterations in 12 $GABA_A$ receptor subunits. After diazepam infusion for 7, 14, or 28 days and 28 days treatment followed by 3 days withdrawal, there were specific subunit changes that were particular to the brain regions examined.

Materials and Methods

Drug Treatment

All animal procedures were approved by the Health Sciences Animal Welfare Committee (University of Alberta) and were in accordance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (180-200g) were housed two per cage, maintained on a 12 hour light-dark cycle (7 am to 7 pm light hours), and acclimatized for a minimum of three days before the start of treatment. Osmotic minipumps deliver diazepam at a relatively constant rate over a 28 day period when 50% DMSO/ 50% PG (v/v) is used as the vehicle (Chapter 2; Arnot et al., 1996); the implantation of these minipumps (Alzet®, Palo Alto, CA) for chronic drug administration was similar to that previously published (Tanay et al, 1996). Briefly, one day before implantation osmotic minipumps were filled either with diazepam in 50% DMSO/50% PG (v/v) vehicle or vehicle alone. Diazepam concentrations were adjusted to deliver 15 mg/kg/day using an IBM Pump Fill protocol (Greenshaw, 1986). The pumps were primed overnight in 0.9% (w/v) saline at room temperature. Prior to surgery, rats were pretreated with a 0.5 ml subcutaneous injection of 2 mg/kg flumazenil, a benzodiazepine antagonist with a short half-life of approximately 10 minutes in rats (Mandema et al., 1991); this pretreatment reduced mortality during and directly following surgery. Rats were anesthetized and maintained with methoxyflurane inhalation. A one to one and a half inch incision was made and osmotic minipumps were subcutaneously implanted in the dorsal thoracic area; wounds were sealed with clips. Animals were monitored daily and the pumps were turned within the subcutaneous pouch every second day. Rats were treated for 7, 14 or 28 days for the chronic dosing paradigm and then sacrificed by decapitation. The animals receiving withdrawal treatment were implanted as above and treated for 28 days. On day 28, the animals were anesthetized as above, an incision made and the pumps removed; wounds were again sealed with clips. On day 31 (3 days following diazepam discontinuation), rats were sacrificed by

decapitation. For all treatment groups the brains were immediately removed, dissected, frozen in liquid nitrogen and stored at -80^{0}C.

Diazepam and flumazenil were generous gifts from Hoffmann-La Roche, Basel, Switzerland.

Quantification of CNS diazepam levels by reversed-phase HPLC

Diazepam levels were quantified as previously described (Holt et al. 1996), with some minor modifications. Cortex (100 mg) was taken from diazepam treated animals, homogenized in five volumes ice cold methanol and centrifuged at 12 000 x g for 20 minutes at 4^{0}C. A 50 µl aliquot of supernatant was injected onto a 25 cm x 0.46 cm Spherisorb C18 column (Alltech, Illinois, U.S.A.) and eluted using a linear gradient of 65% (v/v) to 85% (v/v) methanol with 10 mM potassium phosphate buffer, pH 7, over 15 minutes with a flow rate of 1.0 ml/min. Ultraviolet absorption of the eluant was monitored at 241 nm; flunitrazepam (100 ng/50 µl injection) was used as the internal standard.

RNA Isolation

Total RNA was isolated from specific brain regions using Trizol (Gibco-BRL) and samples were stored at -80^{0}C until required. The quantity and quality of RNA were determined spectrophotometrically (A_{260}/A_{280}) and by agarose gel electrophoresis.

Solution Hybridization

Steady-state mRNA levels were determined using a multiprobe S1 nuclease protection assay (O'Donovan et al., 1991), where the use of multiple length oligonucleotide probes allows simultaneous quantification of several RNA species. This procedure has been previously described in detail (Tanay et al., 1997). Briefly, $GABA_A$ receptor mRNA levels were determined using oligonucleotide probes specific to the large intracellular loop of the individual $GABA_A$ receptor subunits. The specific oligonucleotide probe cDNA

sequences are shown in Table 3.1. The γ2-subunit isoform probe detects both the short and long alternate splice variants of the molecule. Excess ^{32}P-5'-end labeled probe (0.03 pmol for each radiolabeled oligonucleotide probe) was hybridized to 10 or 15 µg total RNA in 30 µl S1 hybridization buffer (0.4 M NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA) at 70^{0}C overnight. Excess non-hybridized probe was digested with S1 nuclease (120 U/ml, Amersham) in S1 nuclease buffer (4.5 mM $ZnSO_4$, 50 mM CH_3COONa pH 4.2, 0.3 M NaCl) for 25 minutes at 37^{0}C. The digestion was terminated by the addition of 300 µl of the reaction mixture to 48 µl S1 stop buffer (4 M CH_3COONH_4, 0.1 M EDTA pH 8.0). Protected (hybridized) oligonucleotide probes were denatured and separated by electrophoresis through a 10% (w/v) polyacrylamide gel (9.88% acrylamide/0.12% bisacrylamide). The internal standard was a probe specific for β-actin mRNA (Table 3.1). The specific activities of the probes were adjusted by dilution with unlabelled probe to ensure that the band intensities were within the linear range for detection (see Appendix 1).

Densitometry

Gels were dried, without fixing, onto a cellophane membrane (Model 543 Gel dryer cellophane backing membrane, BIO-RAD) and images were captured by apposing the gel to a BAS-IIIs Fuji imaging plate (see Appendix 1). Phosphorimaging profiles were created using BAS 1000 MACBAS software with a Bio-imaging analyzer (Fujix) and subsequently analyzed using NIH Image software (Wayne Rasband, RSB, NIMH, NIH, Bethesda, MD). Values were obtained by subtracting the background intensities and integrating the area of the peak. Peak areas were proportional to band intensity, equivalent to the amount of radioactivity which corresponds to protected mRNA species. The linearity and specificity of phosphorimaging and the variance of NIH Image analysis measurements have been determined (Appendix 1). Specific subunit band intensities were normalized to the band intensity of the β-actin internal standard probe and this ratio corresponds to the relative levels of mRNA for each of the subunit isoforms.

Statistical Analysis

Mean relative mRNA levels were normalized to the vehicle treated controls for that subunit isoform and the mean values within a subunit class were compared between the time points. Statistical tests applied the one-way analysis of variance (ANOVA), and if significant changes were demonstrated the Student Newman-Keuls post ANOVA test was performed. Generally animal groups ranged in numbers from 6-12 and in some instances (the cortex) determinations were repeated 2-3 times. There were no inter-assay differences between separate experiments, and this allowed the data sets to be combined (see Appendix 1. Statistics were performed on results from a single subunit class because as the abundance levels of mRNA isoforms change, the variance of measurements for a given subunit changes.

Results

Drug Levels

The cortical diazepam levels were quantified after the various treatment periods. Osmotic minipumps *in vitro* have been demonstrated to deliver diazepam at a relatively constant rate when 50% DMSO/50% PG (v/v) was used as the vehicle (Chapter 2). HPLC analysis of cortices from initial time points during diazepam pump infusion indicate that within 12 hours there was a relatively constant diazepam level (Chapter 4, Figure 4.2). After 7, 14 and 28 days of treatment, the mean concentration of diazepam in rats receiving drug by infusion was 156.4±4.6 ng/g (mean ± SEM; n=8), 118.6± 17.3 (n=12) ng/g and 115.7±6.7 (n=5) ng/g cortex, respectively. Diazepam levels could not be detected in animals that were treated for 28 days and withdrawn for 3 days (< 30 ng diazepam/g cortex).

Steady-State mRNA Changes

The results reported here are only for those subunits that underwent a significant temporal change. The mean steady-state mRNA levels for all subunit isoforms examined are reported in Tables 3.2-3.4.

Cortex:

The data for 14 day cortical mRNA levels are also presented in Chapter 4. There were statistically significant differences amongst the steady-state mRNA levels for α4-, β2, β3-, γ2- and γ3-subunit isoforms during the time course of diazepam infusion (Table 3.2). There was a significant decline in α4-subunit mRNA levels after 7 ($P<0.001$), 14 and 28 ($P<0.01$) days of treatment relative to vehicle controls (Figure 3.1). This decrease in expression returned to levels similar to the vehicle controls after 3 days of withdrawal from diazepam which was significantly different to the initial 7 day treatment level ($P<0.01$). Further, the α4 mRNA level at 7 days was significantly different to the mRNA levels at 14 days of diazepam infusion ($P<0.01$).

There was a time dependent decrease in β2-subunit isoform expression at day 7 which was significantly different from the mean values at day 14 ($P<0.05$) and day 28 ($P<0.01$). However, by day 28, the mean steady-state β2 mRNA levels increased and were significantly different to vehicle control values ($P<0.05$; Table 3.3 and Figure 3.2). The alteration in β3-subunit mRNA levels was significantly increased upon withdrawal of diazepam relative to 7 day and vehicle control values ($P<0.05$; Figure 3.2).

The γ2-subunit expression increased after 28 days treatment, which was significantly different from the 7 and 14 day ($P<0.001$) and vehicle controls ($P<0.01$). After withdrawal, the increased mRNA levels were maintained and were significantly different from vehicle control ($P<0.05$) and 7 and 14 day ($P<0.01$) values (Figure 3.3). The level of γ3-subunit expression was slightly increased at day 14 yet decreased upon diazepam cessation ($P<0.01$ versus day 14 levels; Figure 3.3).

Cerebellum:

There was a significant decrease in the α2-subunit mRNA levels with diazepam infusion and at 7 days this decrease was significant relative to vehicle controls ($P<0.05$; Table 3.3 and Figure 3.4). The levels of α4- and α5-subunit gene expression were also decreased with chronic diazepam treatment. The α4-subunit steady-state mRNA levels were significantly decreased at day 7, 14 ($P<0.01$) and 28 ($P<0.05$) relative to vehicle controls; however, after 3 days cessation from diazepam, the α4-subunit levels rebounded above control values; this was distinct from other treatment groups ($P<0.001$). The α5-subunit mRNA levels were reduced during treatment and withdrawal, and at days 28 ($P<0.05$) and 31 ($P<0.01$), were significantly different from vehicle controls.

The β2- and β3-subunit mRNA levels were significantly increased after 14 days of treatment relative to the other treatment groups ($P<0.05$ for β2-subunit and $P<0.001$ for β3-subunit; Figure 3.5), and by day 28 they returned to normal. At day 28, the level of the γ2-subunit isoform was significantly increased relative to all other time points ($P<0.001$; Figure 3.6) and returned to normal control levels upon diazepam withdrawal. The γ3-subunit expression significantly decreased at 7 and 14 days treatment relative to vehicle ($P<0.05$; Figure 3.6) and upon diazepam cessation rebounded to nearly 150% of the vehicle control, which was significant relative to the other treatment groups ($P<0.001$).

Hippocampus:

The β2- and β3-subunit isoform mRNA levels were significantly decreased at day 28 (Table 3.4 and Figure 3.7). The level of β2-subunit gene expression was significantly decreased relative to other treatment groups ($P<0.001$) and returned to normal levels upon diazepam withdrawal. The decrease in the β3-subunit mRNA level at day 28 was significantly different relative to vehicle ($P<0.05$), 14 day ($P<0.01$) and withdrawal ($P<0.05$) treatment groups.

The γ1-subunit isoform mRNA level was significantly decreased with chronic treatment and at day 28 was significantly different to vehicle, 7 and 14 days treatment

($P<0.05$; Figure 3.8). Upon withdrawal of diazepam, the decreased γ1-subunit mRNA level was maintained and this decrease was significant relative only to the vehicle control values ($P<0.05$). At 14 days the γ2-subunit mRNA level increased and was significantly different to vehicle ($P<0.05$; Figure 3.8); however, when diazepam was discontinued the level of γ2-subunit gene expression returned to control values, which was significantly different from the 14 day treatment values ($P<0.05$).

Discussion

The levels of diazepam in the cortex after continuous infusion with 15 mg/kg/day were relatively constant at the different time points (7, 14, and 28 days). These levels ensure that $GABA_A$ receptor occupancy would be equivalent across time, and that all animals received diazepam; therefore, the only differences between the treatment groups was the length of treatment. There was no detectable diazepam in the brains after 3 days cessation of diazepam infusion, and this is consistent with the pharmacokinetics of diazepam (Friedman et al., 1986). Therefore, the $GABA_A$ receptor changes which occurred upon withdrawal were either a result of the delayed effects of chronic 28 day diazepam infusion or diazepam cessation.

This study showed that chronic drug treatment induced alterations in the levels of $GABA_A$ receptor mRNAs which were dependent on the length of drug treatment. This observation has been previously made for certain $GABA_A$ receptor subunits (O'Donovan et al., 1992a,b). Time dependent changes were found for a number of different subunits (Table 3.2-3.4). For instance, the levels of the α4-subunit mRNA in the cortex decreased with diazepam infusion and upon drug cessation returned to normal. Additionally, the levels of the β2-subunit initially decreased, but recovered to approximate vehicle control levels until an increase at 28 days of treatment, which returned to normal after 3 days of

withdrawal. The day at which the largest changes in mRNA levels were achieved was not consistent among subunits; alterations were found at either 7, 14 and 28 days treatment, depending upon the subunit. Moreover, the levels of subunit isoform mRNAs may decrease at one point and increase at another (for example the β2-subunit isoform in the cerebellum, Figure 3.5), or may not change at all. In summary, a complex pattern of changes was found.

In general, specific subunits showed consistent alterations in gene expression with respect to length of drug treatment. Changes were detected consistently in α4-, β2-, β3-, γ2-, and γ3-subunit mRNA levels with drug treatment. In contrast, the levels of mRNA for other subunits (α1, α3, α6, β1) did not change. Perhaps the other subunit isoform genes were more resistant to chronic treatment, or perhaps alterations in their expression occurred at time points which were not examined. Another possibility was that the alterations in certain subunit isoform mRNA levels were minor and with the method of quantification were not detected.

Cessation of diazepam treatment appeared to result in any of the three possible patterns of gene expression: a rebound effect, levels maintained as day 28, or a return to vehicle control values. The cortical α4- and β2-subunit mRNA levels upon withdrawal showed that mRNA levels did, in some instances, return to pre-treatment levels after the drug was removed. However, the cortical β3-subunit isoform demonstrated that upon withdrawal of diazepam mRNA levels may rebound, where upon drug cessation there was a significant increase in mRNA levels relative to 28 day treatment values. The cerebellar α4-subunit gene expression demonstrated decreased levels at 28 days of chronic treatment, but upon discontinuation of diazepam the mRNA level dramatically increased to levels greater than the vehicle controls. The third pattern of gene expression, as a consequence of withdrawal, was expression similar to that at 28 days treatment. This was most clearly demonstrated with the cerebellar α5-subunit or the hippocampal γ2-subunit isoform. However, these results are dependent on the particular time course required for changes in

gene expression to occur. When values were similar to those of day 28, perhaps diazepam effects were still sustained, or perhaps subunit levels did not have time to rebound in all instances. If this study had examined earlier or later time points, other subunit mRNA levels may have been either returned to normal (after rebound), or may have been in the process of passing through normal levels on the way to rebound levels. It is apparent, however, that subunit isoforms were specific in the time course required for alterations. Further the expression of these genes upon drug cessation was subunit dependent.

The work described in this chapter further examined the brain region specificity of changes that were implicated in previous studies (Kang and Miller, 1991; Primus and Gallager 1992; Impagnatiello et al., 1996). For instance, in the cortex and cerebellum, there was a significant increase in β2-subunit mRNA levels at 28 and 14 days respectively, but in the hippocampus, there was no change until day 28 there was a significant decrease in mRNA levels. All β2-subunit mRNA levels were returned to pre-treatment values upon withdrawal. This was similar to the β3-subunit gene expression where at day 14 the cerebellar levels were increased and the hippocampal levels were normal at day 14 and reduced at day 28; however in both brain regions, they returned to normal on discontinuation of diazepam. The cortical β3-subunit mRNA levels were relatively normal until withdrawal when expression increased. The γ3-subunit mRNA species further demonstrated this brain region specificity. The γ3-subunit mRNA level in the cortex was slightly increased after 14 days treatment and then decreased upon discontinuation of diazepam, whereas the cerebellar γ3-subunit isoform gene expression was decreased throughout chronic treatment and upon cessation of drug treatment the level dramatically increased to values above vehicle control.

It is the working hypothesis of this thesis that chronic drug treatment results in concurrent up- and down-regulation of $GABA_A$ receptor subunits, which in an attempt to compensate for chronic therapy, alters the expression of specific genes, to maintain GABAergic homeostasis. The rebound effect that was demonstrated by subunits during

drug cessation may be subsequently responsible for behavioral characteristics of the withdrawal or rebound phenomena. It may be that the $GABA_A$ receptor system, during compensation, was exposed when the drug was suddenly removed. This over-regulation of subunit gene expression may induce a disproportionate production of protein products and the altered $GABA_A$ receptor may be responsible for the observed changes in behavior. This form of compensation has been previously implicated in behavioral studies of withdrawal and has been termed oppositional tolerance (Chapter 1 and Chapter 5).

This study also demonstrated the transient nature of alterations in $GABA_A$ receptor gene expression. These time course and brain region specific changes are important with respect to interpretation of the current discrepancies in the literature. When comparing different studies, one must keep in mind the number of different variables involved. This chapter confirms that the time points which are examined in a specific study may determine the level of subunit expression. Previous studies with both antipsychotics (Buckland et al., 1992) and benzodiazepines (Kang and Miller, 1991; O'Donovan et al., 1992a,b) have shown that alterations in mRNA levels are specific to the subunit and to the length of treatment. This chapter further confirms initial brain region specificity studies which examined the expression of some subunits in rats (Primus and Gallager, 1992) and mice (Kang and Miller, 1991). In particular, gross anatomical dissections may be responsible for the lack of distinct or adequate detection of alterations in $GABA_A$ receptor gene expression (Impagnatiello et al., 1996) and function (Poisbeau et al., 1997). It is of interest to note that many alterations in $GABA_A$ receptor mRNA levels reported in this chapter do not correlate with previous studies (Heninger et al., 1990; Primus and Gallager, 1992; Wu et al., 1994b; Brett and Pratt, 1995; Holt et al., 1996; Impagnatiello et al., 1996). However, this may result not only from the differences in treatment times, or brain regions examined, but the methods of the dosing paradigm. The importance of the drug regime on the alterations in gene expression are examined in Chapter 4.

The importance of time-course and brain region specificity plays an considerable role in the downstream effects of gene expression and subsequently protein level. A time lag between changes in mRNA levels and subsequent alterations in protein products suggests that although mRNA levels may have returned to normal, alterations in protein expression may still be occurring. Further, it is important to understand that tolerance and withdrawal are time dependent phenomena, where the time course for behavioral alterations may parallel certain alterations in gene expression. If there were no correlations between treatment time and changes in gene expression, one would not expect $GABA_A$ receptor gene expression to play a role in the development of dependence.

In conclusion, the data in this chapter reveal time dependent alterations in $GABA_A$ receptor subunit isoform mRNA levels which were not only subunit specific but also brain region dependent. After diazepam discontinuation, the alterations in gene expression were either maintained or rebounded. The diazepam discontinuation was examined at 3 days post-infusion and it is possible that some alterations in $GABA_A$ receptor subunit gene expression may be altered at earlier or later time points. However, the exact significance of these $GABA_A$ receptor subunit gene expression alterations in behavioral symptoms of withdrawal are unknown because the behavioral correlates were not concurrently examined. Nonetheless, based on the reported temporal development of benzodiazepine dependence, generalizations regarding these $GABA_A$ receptor mRNA levels and behavioral tolerance and withdrawal may be applied.

Transcript Specificity	Oligonucleotide Sequence	Nucleotides	Reference
α1	5'>GGGGTCACCCCTGGCTAAGTTAGGGGTATA GCTGGTTGCTGTAGG<3'	1166-1210	Khrestchatisky et al., 1989
α2	5'<AGATTCGGGGCGTAGTTGGCAACGGCTACA GCA<3'	1445-1477	Khrestchatisky et al., 1991
α3	5'<CTCAGCAGGACTGTCTTGCACATAAGTGGT CTTGGGGGAAGCAACACTG<3'	1533-1582	Malherbe et al., 1990
α4	5'<CAAGTCGCCAGGCACAGGACGTGCAGGAG G<3'	57-86	Wisden et al., 1991
α5	5'<CACAGCATTCCCAGTCCCGCCTGGAAGCTG CTCCTTTGGGA<3'	1485-1525	Malherbe et al., 1990
α6	5'<CGTTGATGGTAAGATGGGCGTTCTACTGAG GACTTTGCTGGCCTCAGAAGATGGAACGAT<3'	1141-1200	Luddens et al., 1990
β1	5'<ATGGCAACCATCACAGGAAAAGAGAGAAG CCCCAAACTCTCTCGA<3'	95-139	Ymer et al., 1989
β2	5'<TCGTTCCAGGGCGTTGCGGCCAAAACTATG CCTAGGCAACC<3'	96-136	Ymer et al., 1989
β3	5'<CTGAATTCCTGGTGTCACCAACGCTGCCTG CAACCTCATTCATTTCATTGTGAAC<3'	1190-1244	Ymer et al., 1989
γ1	5'<GCAGTCTTCAAAGCAACAGAAAAAGGTAGC ACAGTCTTTGCCCTCCAAGC<3'	1217-1266	Ymer et al., 1990
γ2	5'<GTTCATTTGGATCGTTGCTGATCTGGGACGG AT<3'	1183-1215	Shivers et al., 1989
γ3	5'<AGAGGGTGCTTAAGGCTTATTCGCTCAGGA ATCCATCTTGTTGAATCTGGATGT<3'	1170-1224	Herb et al., 1992
β-actin	5'<CTGGTGGCGGGTGTGGACCGGGACGGAGGA GCTGCAA<3'	272-308	Nudel et al., 1983

Table 3.1:

The sequences and positions (within the given references) of the oligonucleotide probes which were used in the S1 nuclease assay to quantify $GABA_A$ receptor steady-state mRNA levels.

Time course of cortical steady-state mRNA levels as a percentage of vehicle (mean±SEM; n)					
Subunit	Vehicle	7 days	14 days	28 days	28 days & withdrawal
α1	100±2.0 (22)	97.7±5.5 (16)	102.0±1.6 (33)	99.8±5.2 (6)	101.8±5.6 (9)
α2	100±2.4 (17)	98.0±4.2 (20)	102.0±3.3 (14)	92.0±4.1 (6)	103.3±10.2 (9)
α3	100±4.3 (19)	107.6±2.6 (19)	99.2±3.6 (25)	98.4±2.9 (6)	96.2±3.8 (8)
α4	100±4.8 (18)	60.3±4.1 (13) ***[b,d]	79.6±2.9 (16) **	75.5±13.2 (5) **	92.9±3.8 (5)
α5	100±1.7 (19)	104.0±4.9 (19)	110.1±2.0 (15)	105.9±9.7 (6)	100.0±8.4 (9)
β1	100±3.0 (16)	94.1±4.6 (16)	121.0±10.5 (15)	115.1±14.3 (6)	126.5±14.3 (8)
β2	100±3.8 (20)	94.1±4.7 (18) *	109.8±3.5 (19)[a]	122.6±9.5 (6) *[a]	107.2±3.4 (9)
β3	100±3.3 (19)	100.4±5.6 (19)	104.9±4.8 (21)	97.5±5.3 (6)	126.4±9.2 (8)*[a]
γ1	100±4.6 (25)	109.5±4.5 (23)	133.3±15.0 (16)	97.7±9.6 (6)	111.4±12.0 (8)
γ2	100±3.4 (24)	90.3±5.6 (22)	90.8±3.7 (21)	140.5±20.4 (6)**[a,b]	132.5±1.5 (5)*[a,b]
γ3	100±4.5 (16)	101.5±4.9 (12)	116.6±8.5 (17)	110.3±8.5 (6)	71.8±9.3 (8) [b]

Table 3.2:

The mean cortical $GABA_A$ receptor steady-state mRNA levels as a percentage of vehicle treated controls following 15 mg/kg/day diazepam infusion for 7, 14, 28 days and 28 days plus 3 days withdrawal. The mean and SEM values were calculated from separate S1 nuclease assays performed on 6-12 animals (n) per treatment group. For instance where the n values were greater than 12, the assay was repeated. mRNA levels were normalized to the β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ relative to vehicle controls. "a" represents significance relative to 7 day, "b" relative to 14 day, and "d" relative to withdrawal treatment groups. Statistical analysis was performed using a one-way ANOVA followed by the Student Newman-Keuls post ANOVA test.

Time course of cerebellar steady-state mRNA levels as a percentage of vehicle (mean±SEM; n)					
Subunit	Vehicle	7 days	14 days	28 days	28 days & withdrawal
α1	100±6.0 (9)	86.9±6.3 (10)	90.6±2.5 (7)	96.3±5.1 (6)	97.5±5.6 (8)
α2	100±12.5 (7)	63.7±4.0 (10)*	91.0±5.5 (8)	72.7±11.4 (5)	70.9±9.5 (5)
α3	100±8.2 (6)	107.1±7.8 (10)	113.7±6.8 (10)	83.7±4.7 (4)	117.5±6.6 (5)
α4	100±8.2 (6)	61.1±7.1 (8)**	70.0±6.1 (8)**	66.7±11.5 (5)*	166.1±13.7 (5)***a,b,c
α5	100±9.6 (8)	73.3±8.1 (10)	80.9±8.8 (7)	47.5±7.5** (5)	50.6±15.2* (5)
α6	100±6.0 (8)	84.4±7.1 (9)	114.3±7.9 (7)	92.2±7.9 (6)	98.3±4.0 (7)
β1	100±10.4 (8)	105.2±3.7 (9)	76.7±8.5 (5)	126.1±9.5 (5)	106.1±10.1 (7)
β2	100±5.9 (10)	97.5±8.9 (11)	128.0±9.3 (7)*a,b,d	101.6±6.5 (6)	100.0±3.3 (5)
β3	100±7.3 (7)	79.7±11.1 (5)	174.0±23.7 (6)***a,b,d	103.9±8.2 (6)	97.5±5.5 (10)
γ1	100±6.4 (5)	98.8±13.3 (6)	127.1±18.7 (6)	135.4±15.6 (5)	92.7±4.8 (8)
γ2	100±7.8 (9)	100.6±4.6 (8)	93.9±6.4 (7)	167.3±13.8 (5) ***a,c,d	110.0±10.2 (10)
γ3	100±8.8 (5)	67.5±1.3 (6)*	68.6±2.8 (9)*	77.6±10.9 (5)	148.6±17.3 (5)***a,b,c

Table 3.3:

The mean cerebellar $GABA_A$ receptor steady-state mRNA levels as a percentage of vehicle treated controls following 15 mg/kg/day diazepam infusion for 7, 14, 28 days and 28 days plus 3 days withdrawal. The mean and SEM values were calculated from separate S1 nuclease assays performed on 6-12 animals (n) per treatment group. mRNA levels were normalized to the β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ relative to vehicle controls. "a, b, c, and d" represent significance relative to 7, 14, 28 day, and withdrawal groups, respectively. Statistical analysis used a one-way ANOVA followed by Student Newman-Keuls post ANOVA test.

Time course of hippocampal steady-state mRNA levels as a percentage of vehicle (mean±SEM; n)					
Subunit	Vehicle	7 days	14 days	28 days	28 days & withdrawal
α1	100±3.8 (12)	95.3±5.5 (12)	95.5±4.7 (8)	76.7±13.8 (5)	92.5±4.3 (9)
α2	100±2.5 (12)	78.3±10.9 (7)	114.3±6.7 (7)	101.4±9.6 (5)	96.9±12.2 (7)
α3	100±6.9 (8)	94.4±8.6 (10)	100.8±7.5 (8)	115.8±18.6 (6)	79.6±7.2 (8)
α4	100±6.8 (7)	110.3±13.5 (9)	70.2±7.6 (9)	85.8±6.5 (8)	89.4±6.7 (9)
α5	100±4.8 (7)	98.7±5.1 (11)	95.3±3.0 (9)	101.8±4.5 (8)	83.2±6.1 (9)
β1	100±9.8 (9)	100.6±11.2 (8)	86.6±7.6 (7)	83.3±6.0 (5)	97.4±11.4 (10)
β2	100±5.2 (9)	103.2±6.8 (9)	102.5±7.2 (9)	46.6±5.1 (6)$^{***.a,b,d}$	86.9±6.6 (10)
β3	100±3.9 (9)	86.8±4.6 (8)	106.5±7.7 (8)	69.4±6.5 (5)$^{**.b,d}$	93.6±6.3 (9)
γ1	100±5.7 (7)	93.8±2.7 (9)	95.2±2.6 (9)	67.0±9.2 (5)$^{*.a,b,c}$	76.2±8.5 (9)*
γ2	100±10.4 (8)	129.9±12.3 (6)	143.4±13 (7)$^{*.d}$	136.7±14 (5)	89.2±10.5 (6)
γ3	100±4.9 (7)	89.9±2.8 (7)	89.7±6.7 (8)	94.0±14 (5)	88.0±10.3 (9)

Table 3.4:

The mean hippocampal $GABA_A$ receptor steady-state mRNA levels as a percentage of vehicle treated controls following 15 mg/kg/day diazepam infusion for 7, 14, 28 days and 28 days plus 3 days withdrawal. The mean and SEM values were calculated from separate S1 nuclease assays performed on 5-12 animals (n) per treatment group. mRNA measurements were normalized to the β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ relative to vehicle controls. "a, b, c, and d" represent significance relative to 7, 14, 28 day, and withdrawal groups, respectively. Statistical analysis used a one-way ANOVA followed by Student Newman-Keuls post ANOVA test.

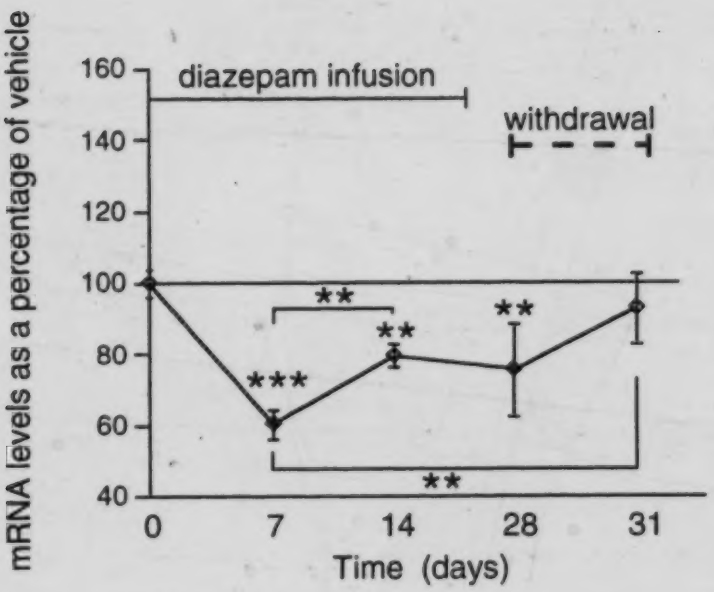


Figure 3.1:

The time course of mean cortical α4-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. The 7 day time point was significantly different from vehicle (***), 14 (b) and 31 days (d). Further the 14 and 28 day treatments were significantly different from the vehicle controls (**). ** represents $P<0.01$, b represents $P<0.01$ relative to 14 day, d represents $P<0.01$ relative to withdrawal and *** represents $P<0.001$ relative to vehicle using a one-way ANOVA followed by Student Newman-Keuls.

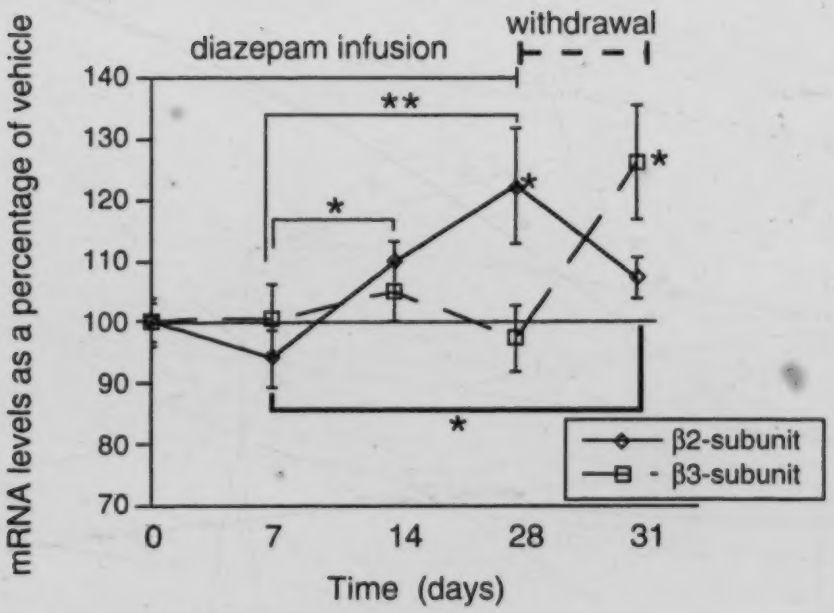


Figure 3.2:

The time course of mean cortical β2- and β3-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. For the β2-subunit isoform, the 28 day level was significantly different from both the 7 day (**) and vehicle levels (*); 14 days was also significant relative to the 7 day treatment (*). For the β3-subunit isoform, the withdrawal mRNA levels were significantly increased (*) relative to the 7 day (thick line) and vehicle levels (*). * represents $P<0.05$ and ** represents $P<0.01$ using a one-way ANOVA followed by Student Newman-Keuls.

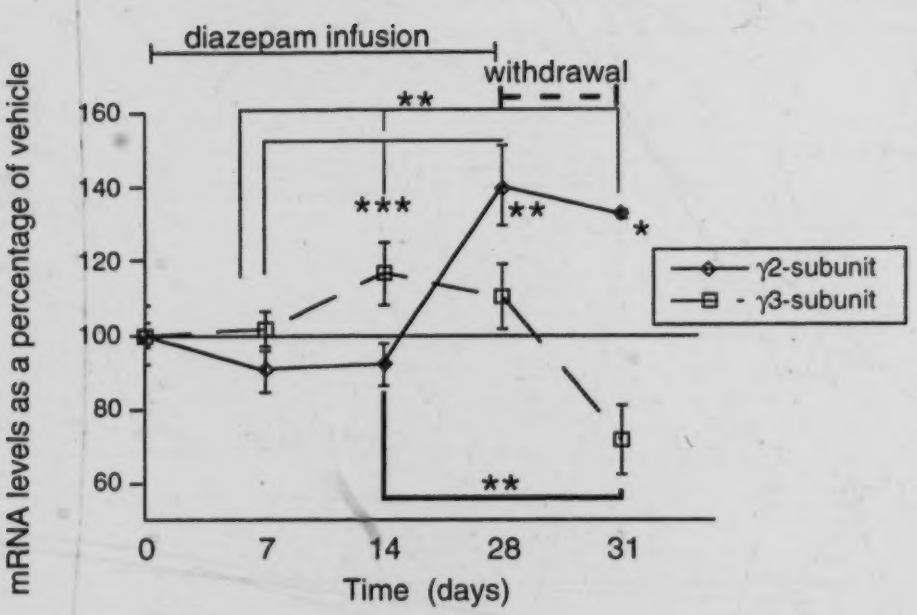


Figure 3.3:

The time course of mean cortical γ2- and γ3-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. For the γ2-subunit isoform the vehicle level was significantly different from the 28 day (**) and withdrawal (*) treatments. These time points were also different relative from the 7 and 14 day time courses (** versus 31 day and *** versus 28 day). For the γ3-subunit isoform, the withdrawal levels were significantly decreased (**) relative to 14 day levels (thick line). * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

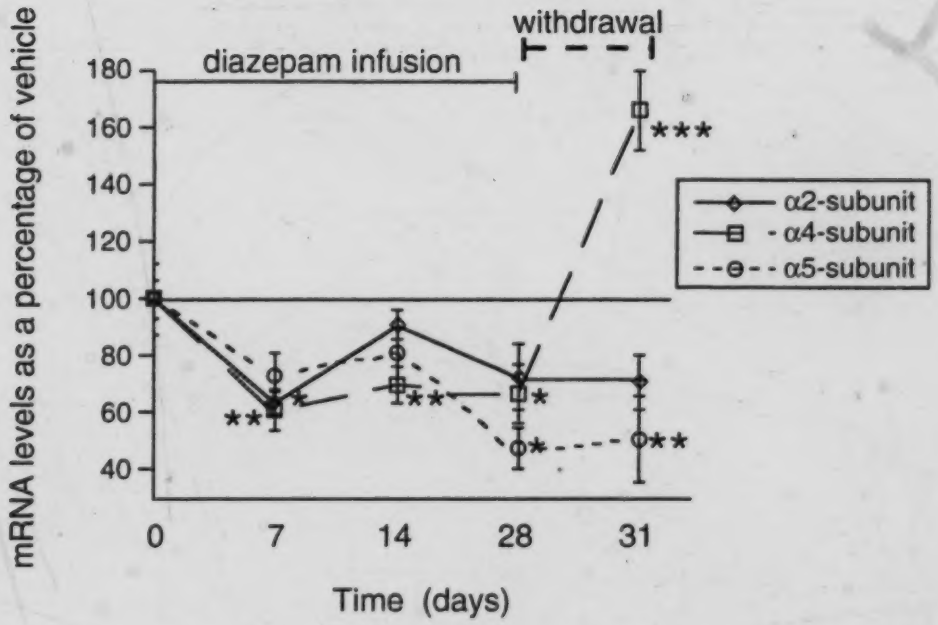


Figure 3.4:

The time course of mean cerebellar α2-, α4- and α5-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. For the α2-subunit isoform, the 7 day level was significantly different from the vehicle controls (*). For the α4-subunit mRNA levels, withdrawal was significantly increased relative to all other time points (***). Further the 7, 14 ( ) and 28 (*) day values were different from vehicle. For the α5-subunit isoform, the 28 day (*) and withdrawal (**) levels were significantly decreased relative to vehicle controls. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

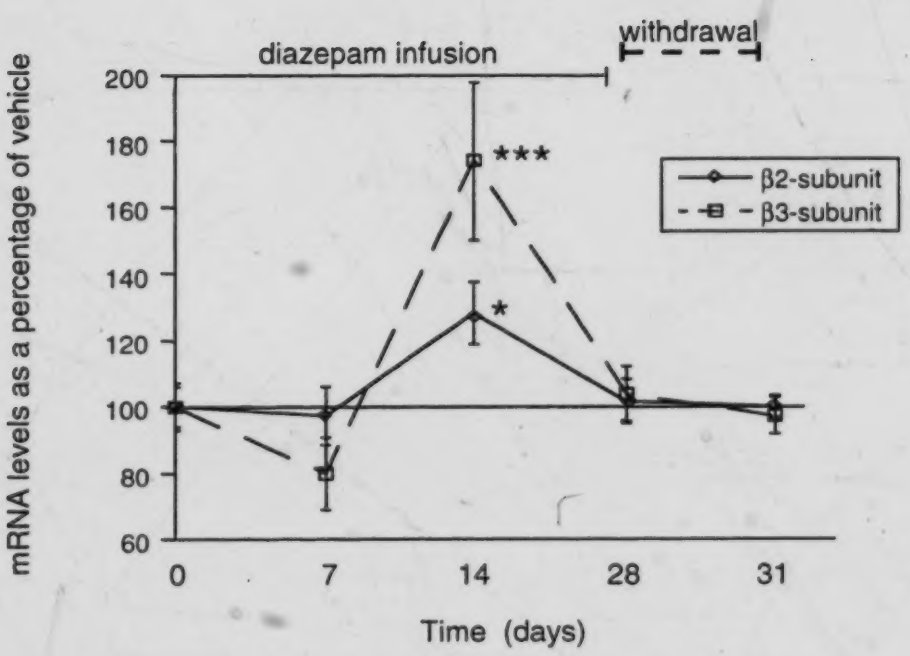


Figure 3.5:

The time course of mean cerebellar β2- and β3-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. For both the β2- and β3-subunit isoforms, the 14 day levels were significantly increased relative to the vehicle, 7 day, 28 day and withdrawal groups. * represents $P<0.05$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

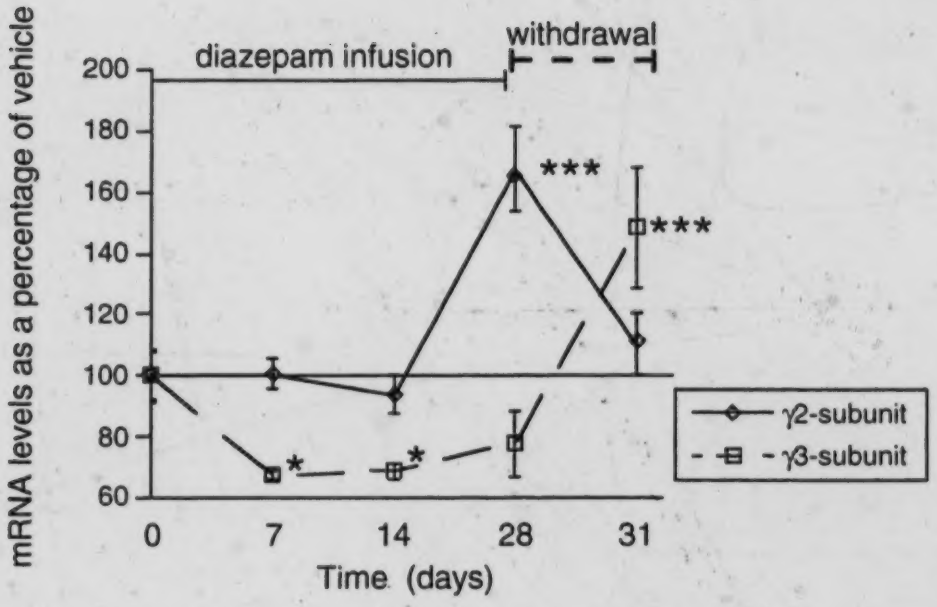


Figure 3.6:

The time course of mean cerebellar γ2- and γ3-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. For the γ2-subunit isoform, the 28 day level was significantly increased (***) relative to the vehicle, 7 day, 28 day and withdrawal groups. The γ3-subunit isoform levels were significantly decreased (*) relative to vehicle at 7 and 14 days treatment; however, on withdrawal, the level significantly increased relative to all others (***). * represents $P<0.05$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

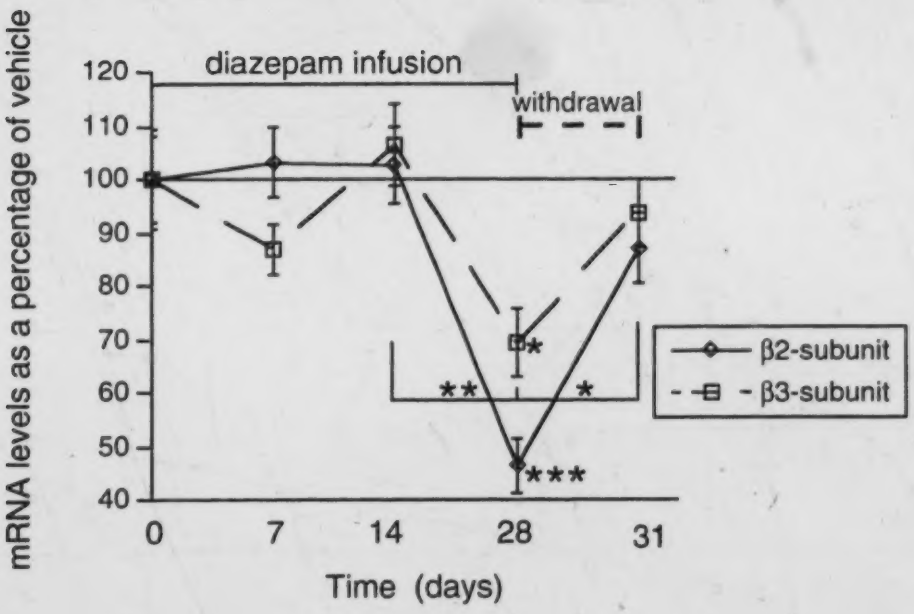


Figure 3.7:

The time course of mean hippocampal β2- and β3-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. The β2-subunit isoform at day 28 was significantly less than all other time points (***). The 28 day level of the β3-subunit isoform was significantly different from 14 day (**) and the vehicle and withdrawal groups (*). * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

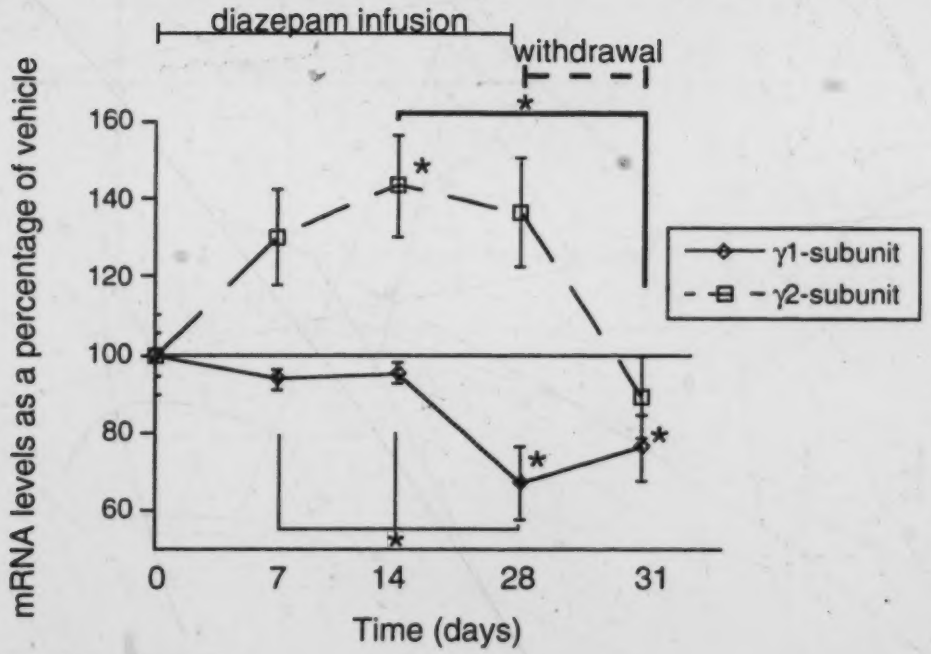


Figure 3.8:

The time course of mean hippocampal γ1- and γ2-subunit steady-state mRNA levels as a percentage of vehicle controls after continuous infusion and 3 days withdrawal from 15 mg/kg/day diazepam. Error bars represent SEM calculated from independent S1 nuclease assays performed on 6-12 animals per treatment group. mRNA levels were normalized to the β-actin internal standard. The γ1-subunit isoform at day 28 was significantly less than the vehicle, 7 and 14 day time points (*) and the withdrawal level was significantly lower than the vehicle controls (*). The 14 day level of the γ2-subunit isoform was significantly increased relative to the vehicle and withdrawal groups (* and thick line). * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one-way ANOVA followed by Student Newman-Keuls.

CHAPTER 4

$GABA_A$ Receptor Isoform Gene Expression in Rat Cortex: Effects of Chronic Diazepam Exposure via Two Different Treatment Regimes

Introduction

Diazepam is a widely prescribed benzodiazepine, yet with daily use tolerance may develop to particular aspects of its pharmacological profile, most noticeably its sedative and anticonvulsant effects (Chapter 1; File and Pellow, 1990). The molecular mechanisms which underlie benzodiazepine tolerance are not understood.

Chronic treatment with diazepam or other full agonist benzodiazepines has been shown to alter: $GABA_A$ receptor affinity for the agonist GABA, functional sensitivity, bicuculline seizure threshold and some benzodiazepine binding characteristics (Gallager et al., 1984a,b; 1985; Yu et al., 1988; Hernandez et al., 1989; Marley and Gallager, 1989; Tietz et al., 1989; Wu et al., 1994a). It has been hypothesized that chronic benzodiazepine treatment may alter $GABA_A$ receptor gene expression and that it is this process which underlies subsequent alterations in benzodiazepine binding capacity and the development of tolerance. Diazepam has previously been shown to modify $GABA_A$ receptor steady state mRNA levels, although the results have not been entirely concordant between laboratories (Heninger et al., 1990; Primus and Gallager, 1992; Wu et al., 1994b; Brett and Pratt, 1995; Holt et al., 1996; Impagnatiello et al., 1996). For example, after chronic diazepam treatment, the $GABA_A$ receptor mRNA levels for the α1-subunit isoform have been shown to be either significantly decreased (Holt et al., 1996) or depending on the cortical localization unaltered and increased (Brett and Pratt, 1995). Similar incongruities in results have also been reported for benzodiazepine binding (Rosenberg and Chiu, 1981; Gallager et al, 1984b; Hernandez et al., 1989; Tietz et al., 1989; Wu et al., 1994a; Brett and Pratt, 1995). The discord in the literature may be due to several factors: the acuity of the techniques used to measure the mRNA levels (reverse transcriptase PCR, Northern blot, *in situ* hybridization, or solution hybridization), the anatomical areas examined (whole brain or specific brain regions), the dosage administered, or the methods of drug treatment (intraperitoneal or depot injection, pump infusion, or p.o.). Some parameters have been

explored, for example, changes in specific subunit gene expression are clearly brain region specific (Chapter 3; Primus and Gallager 1992; Impagnatiello et al., 1996). Some parameters (drug treatment, radioligand utilized for characterization) may also be responsible for varying results in binding analysis. In this study, two diazepam treatment paradigms differentially alter the expression of $GABA_A$ receptor genes and the binding characteristics of receptors subsequently expressed. Exposure of rats for 14 days to the same daily dose of diazepam, delivered either by single daily injection or by constant infusion via osmotic minipumps, produced significantly different changes in specific $GABA_A$ receptor subunit mRNA levels, and consequently, resulted in atypical $GABA_A$ receptor benzodiazepine binding and modulation.

Materials and Methods

Drug Treatment

Animals were acclimitized and housed as described previously (Chapter 3). The procedure for implantation and chronic administration with osmotic minipumps (Alzet®, Palo Alto, CA) was as described in Chapter 3. The second treatment paradigm utilized a group of rats which were subcutaneously (s.c.) injected daily with either 15 mg/kg diazepam in 50% DMSO/50% PG (v/v) vehicle or vehicle alone. (The injection volume was 1 ml/300g rat.) All rats were treated for 14 days, sacrificed by decapitation 22-24 hours after the last dose and the brains immediately removed, dissected, frozen in liquid nitrogen and stored at -80°C.

Diazepam was a generous gift from Hoffmann-La Roche, Basel, Switzerland.

Diazepam quantification, RNA isolation, solution hybridization and densitometric analysis

These methods of biochemical preparation, quantification and analysis were described previously in Chapter 3 and Appendix 1.

Binding Analysis

Cortical tissue from sacrificed animals was washed and homogenized in 50 volumes ice-cold 50 mM Tris-HCl (pH 7.4). Briefly, tissue was homogenized, incubated on ice for 10 minutes and then centrifuged at 12 000 x g for 30 minutes at 4^0C, and pellets resuspended in 50 volumes ice cold 50 mM Tris-HCl (pH 7.4). This procedure was repeated for a total of 5 times. Final pellets were resuspended in 20 volumes 50 mM Tris-HCl pH 7.4, and protein levels were adjusted to 0.75 mg/ml. Protein concentrations were determined using standard BIORAD BSA assay. Each binding assay was carried out in a final volume of 1 ml 50 mM Tris-HCl (pH 7.4), containing 100 µl tissue and the radioactive species. Each assay was incubated for 2 hours at 4^0C; all assays were performed in triplicate. Bound and free ligand were separated by filtration using a Brandel cell harvester, and the GF/B filters were washed twice with 4 ml of ice-cold Tris-HCl buffer. To ensure protein integrity of the tissue prior to binding experiments, long-term storage was at -80^0C.

Benzodiazepine Receptor Subtype Definition

A final concentration of 2 nM [^{3}H]-flunitrazepam (83.4 Ci/mmol; Dupont-NEN) was displaced with either 100 nM or 5 µM zolpidem. Non-specific binding was defined with clonazepam (2 µM). Based on the affinity of zolpidem for $GABA_A$ receptors comprised of various α subunits, benzodiazepine receptor subtypes could be discriminated (Figure 4.1). Zolpidem was a generous gift from Synthelabo, France.

GABA Enhancement

Cortical issue was incubated with 75 mM NaCl, and 2 nM [^{3}H]-flunitrazepam (83.4 Ci/mmol; Dupont-NEN). Enhancement of binding was achieved by the addition of 100 μM GABA , and non-specific binding was defined using 2 μM clonazepam.

Total Specific Binding and Diazepam Insensitive Binding

Total specific binding was defined by incubating a final concentration of 10 nM [^{3}H]-Ro 15-4513 (21.7 Ci/mmol; Dupont-NEN) in the presence of 10 μM cold Ro 15-4513. Diazepam insensitive binding was defined by the addition of 10 μM cold diazepam, which was pre-incubated with the tissue/buffer solution for 30 minutes prior to the addition of [^{3}H]-Ro 15-4513. Ro 15-4513 was a generous gift from Hoffmann-La Roche, Basel, Switzerland.

Statistical Analysis

Mean relative mRNA levels were normalized to vehicle treated controls and compared between treatment groups within a subunit class. Statistical tests applied included the *F*-test to determine which groups had equal variances, followed by the appropriate unpaired Student's *t*-test. Generally animal groups ranged in numbers from 4-6 and determinations were repeated 3-4 times. There were no inter-assay differences between separate experiments, and this allowed the data sets to be combined (see Appendix 1). Statistics were performed on results from a subunit isoform class because as the abundance levels of mRNA subunit isoforms change, the variance and accuracy of measurements for a given subunit family changes.

For GABA enhancement and benzodiazepine subtype ratios, statistical analysis was performed using a one way analysis of variance (ANOVA), between tissue from diazepam injected, diazepam infused and vehicle treated animals; the post ANOVA test performed was the Student Newman-Keuls. To compare the diazepam treatment regimes with respect

to total specific Ro 15-4513 binding and diazepam insensitive binding, an unpaired Student's *t*-test was performed. The sample number (n) represents the number of animals from which tissue was obtained.

Results

Drug Levels

After 14 days of chronic treatment the mean concentration of diazepam in rats receiving drug by infusion was 118.6 ± 17.3 (mean ± SEM; n=12) ng/g cortex (Chapter 3) and in rats treated by daily s.c. injection 106.3 ± 9.3 (n=9) ng/g cortex 22-24 hours after the last injection. Osmotic minipumps *in vitro* deliver diazepam at a relatively constant rate when 50% DMSO/50% PG (v/v) is used as the vehicle (Chapter 2). Further, HPLC analysis of early time points from cortex of animals receiving diazepam from osmotic minipumps (15 mg/kg/day) indicates similar brain levels post implantation (Figure 4.2). The largest deviation in levels occurs between the 24 hour time point (92.2 ± 8.1 ng/g), and the 96 hour time point (159.2 ± 14.7 ng/g). A single daily s.c. injection (15 mg/kg diazepam) results in an initial increase in diazepam levels in the cortex (Figure 4.2), with the maximum measured concentration occurring 2 hours after injection (398.2±30.6 ng/g) followed by a decline to the limit of detection 24 hours post injection (33.8±2.4 ng/g).

Changes in Steady-State mRNA Levels

The cortical steady-state mRNA levels from pump infusion animals have previously been reported in Chapter 3 of this thesis, but for continuity are included in this chapter as well.

Differences Between Drug and Vehicle Treatment

In the daily injected treatment group there was a significant increase in the α3-, α4- and β3-subunit steady-state mRNA levels and a significant decrease in the α1- and β2-subunit steady-state mRNA levels compared to their respective vehicle treated controls. In the pump infused treatment group there was a significant increase in the α5-, β2-, and γ1-subunit gene expression, and a significant decrease in the α4- and γ2-subunit gene expression relative to their vehicle treated controls (Table 4.1).

Differences Between Diazepam Treatment Regimes

α1-, α3- ($P < 0.05$) and α4- ($P < 0.001$) subunit $GABA_A$ receptor mRNA levels were significantly different between daily diazepam injection and diazepam pump infusion treatment groups (Figure 4.3). Animal groups that were injected daily versus pump infused had mean steady-state mRNA levels for the α1-subunit of 96.1±2.1% (n=13, where n= number of determinations) and 101.2±1.6% (n=33), for the α3-subunit of 108±3.7% (n=19) and 99.2±3.6% (n=25), and for the α4-subunit of 117.9±7.7% (n=14) and 79.6±2.9% (n=16), respectively. Further, differences were also found between treatment regimes in the $GABA_A$ receptor β2- ($P < 0.001$), β3-, and γ1- ($P < 0.05$) subunit mRNA levels (Figure 4.4). The mean steady-state mRNA levels of these subunits between injection and pump infusion were 91.6±3% (n=12) and 109.8± 3.5% (n=19) for the β2-subunit, 122.5±6.9% (n=11) compared to 105±4.9% (n=21) for the β3-subunit, and 95.5±8.3% (n=11) and 133.3±14.9% (n=16) for the γ1-subunit, respectively. There were no significant differences between the treatment groups in the α2-, α5-, β1-, γ2- and γ3-subunit mRNA levels. The low abundance of the α6-subunit mRNA in the cortex did not allow its consistent quantification.

Binding Analysis

There were no significant differences in the BZ2/BZ1 and BZ3/BZ2 ratios in cortical tissue from the vehicle, diazepam injection or infusion treated animals (Table 4.2). However, the GABA enhancement of flunitrazepam binding in tissue from both diazepam injection and pump infusion treatment groups is significantly compromised ($P<0.001$, Figure 4.5), with only 121.3±4.2% (n=5) and 113.8±3.6% (n=6), respectively, compared to the GABA enhancement of 153.8 ± 4% (n=8) in tissue from vehicle controls. There was no significant difference in regard to the magnitiude of [^{3}H]-flunitrazepam binding between treatment regimes and vehicle membrane preparations. Further, all membrane pellets were washed extensively, therefore the probability that residual diazepam confounded results is minimal.

Subsequent modifications in binding characteristics between treatment groups was demonstrated with both specific [^{3}H]-Ro 15-4513 binding and diazepam insensitive (DIS) binding (Figure 4.6 and 4.7). Specific binding of [^{3}H]-Ro 15-4513 was significantly increased in tissue from diazepam injected animals compared to tissue from diazepam pump infused animals ($P<0.01$; 821±82 fmol/mg and 646±13 fmol/mg, respectively, n=6). Further, although there was an increase in the amount of [^{3}H]-Ro 15-4513 binding, significantly less ($P<0.05$) was displaced by the addition of diazepam in the tissue from diazepam injected relative to tissue from diazepam infused animals. The diazepam insensitive binding component relative to total specific [^{3}H]-Ro 15-4513 binding was 2.6 ± 0.3% in the diazepam injected animals compared to 1.6±0.3% in those that received the drug by infusion (n=6, and vehicle DIS binding is 1.9± 0.3%; n=7).

DISCUSSION

The two different dosing schedules provide the same daily doses (15 mg/kg), and after 14 days of either treatment, continuous pump infusion or daily injections, diazepam levels in the cortex were comparable. However, with single daily injections the diazepam concentration in the cortex peaked 2 hours post-injection, declining over the following 22 hours; this results in a marked fluctuation in receptor occupancy by diazepam at the benzodiazepine site on the $GABA_A$ receptor over a 24 hour period. The diazepam elimination half-life in rats has been previously shown to be approximately 1 hour (Friedman et al. 1986), and diazepam is a highly lipophilic and plasma protein bound drug; after a 10 mg/kg intraperitoneal (i.p.) injection, the mean residence time has been reported to be 41 minutes (Zomorodi et al. 1995). Further, at high concentrations of diazepam in the mouse, first order kinetics no longer operate due to saturation of metabolic pathways (St. Pierre and Pand 1995). Previous reports of brain diazepam concentrations both 2 hours after 21 days i.p. 5 mg/kg/day diazepam administration and after 14 days of 15 mg/kg/day depot injection (Holt et al. 1996) were approximately 250 ng/g (Heninger et al. 1990). Further, Gallager and co-workers have reported that 24 hours after 21 days of 5 mg/kg/day i.p. diazepam there was less than 0.01% of the administered dose detected in the brain (Gallager et al. 1984a,b) however, at peak levels 30 minutes following this injection, brain diazepam levels were dramatically higher (approximately 30% of the administered dose; Gallager et al. 1985). Nonetheless, 2 hours after a higher dose of 15 mg/kg via a single s.c. injection, diazepam brain concentrations were 3% (400 ng/g cortex) and 24 hours post injection declined to 0.2% (30 ng/g cortex, the level of detection) of the initial dose administered. This difference in drug levels may be a result of the method of diazepam treatment (s.c. versus i.p.), the lower absorption rate of the s.c. injection or, the saturation of the metabolic paths after the larger 15 mg/kg/day diazepam dose. These mechanisms may limit diazepam distribution in the brain and cause diazepam to be detected

for a prolonged period, as metabolism of the drug has not been as marked. In this study, 24 hours after 14 days of chronic diazepam treatment via either daily s.c. injection or osmotic minipump infusion the brain levels of diazepam detected were similar (0.7% and 0.8% of the daily administered dosage, respectively).

We have demonstrated that *in vitro* Alzet® osmotic minipump infusion delivers diazepam at a relatively constant rate over a 28 day period when 50% DMSO/50% PG (v/v) is used as the vehicle (Chapter 2; Arnot et al. 1996). *In vivo* studies suggest that steady-state levels are reached after 48 hours (Figure 4.2). The differences in receptor occupancy produced by the two dosing regimes may be responsible for the differential alterations in $GABA_A$ receptor subunit mRNA levels and subsequent changes in $GABA_A$ receptor benzodiazepine binding characteristics. The distinct kinetics or fluctuations of the drug at the $GABA_A$ receptor results in the receptor's differential response to the two drug treatments.

After 14 days chronic treatment with 15 mg/kg/day diazepam via daily injection or pump infusion there was a significant difference in levels of $GABA_A$ receptor mRNA for α1-, α3, α4-, β2-, β3-, and γ1-subunits. It has been previously demonstrated that diazepam interacts with $GABA_A$ receptor subtypes that include these subunits, with the exception of the α4 subunit. Indeed, $GABA_A$ receptor subtypes that contain α4 subunits are generally classified as "diazepam insensitive" (Wisden et al., 1991), and therefore alterations in the expression of this subunit isoform may alter the subsequent modulation of the $GABA_A$ receptor by diazepam.

The magnitude of the changes differs markedly between the α-subunit isoform mRNAs; this may be rationalized by the differences in the relative abundance of specific $GABA_A$ receptor subunit mRNAs within the cortex. Based on oligonucleotide specific activities required to generate equivalent band intensities, the α1-subunit steady-state mRNA levels in normal rat cortex are approximately 10 times greater than those of the α4 subunit (the lowest abundance α subunit detected in the cortex), 5 times greater than those

for the α3 subunit, and 4 times greater than those for the α2 and α5 subunit. For example, in the daily injected treatment group, examination of the α-subunits illustrate how the relative isoform abundance levels may affect $GABA_A$ receptor subtype expression. If the changes in mRNA steady-state levels were normalized, taking into account their relative abundance in the cortex, one can demonstrate that there were no significant differences between the decrease in the sum of α1- and α2-subunit mRNA levels and the sum of the increases in the α3-, α4-, and α5-subunit isoform mRNA levels (Figure 4.8). The small changes seen in the α1- (4% decrease) and α2-(0.7% decrease) subunit mRNA levels could have equivalent consequences to the larger changes found in the levels of the less abundant α3- (8% increase), α4- (18% increase) and α5- (11% increase) subunit mRNAs, assuming that there is a direct relationship between mRNA and protein levels. Accordingly, these data are consistent with the hypothesis that chronic benzodiazepine treatment causes the concomitant up- and down-regulation of the expression of specific $GABA_A$ receptor subunit mRNAs. Further, it suggests that there may be a degree of coordinate regulation in $GABA_A$ receptor subunit gene expression, in response to a chronic diazepam treatment.

To establish if there is a correlation between these changes in $GABA_A$ receptor mRNA levels and protein products, we measured various cortical binding characteristics from experimental animals treated by both dosing regimes. Protein levels can be quantitated by a number of different methods (immunoprecipitation, Western blots, and ligand binding analysis), and although the first two techniques measure protein expression, they are difficult to quantify and the lack of commercially available specific antibodies compromise such a study.

Binding analysis, however is not only quantifiable but also is a "pragmatic measure" of receptor protein expression, in an *in vitro* system. The consequences of altered $GABA_A$ receptor subunit composition may be assessed using benzodiazepine ligands with distinct recognition/binding profiles. Through analysis of diverse benzodiazepine ligand binding and the interaction of benzodiazepines and GABA one can

hypothesize the $GABA_A$ receptor subunit isoform composition. It has been shown previously (Chapter 1; see Sieghart 1995 for review) that binding affinity is altered with changes in the subunit composition, yet this analysis is limited because one must assume that subunits are replaced by isoforms from the same family. The extrapolation of postulated changes in benzodiazepine subtype characteristics, defined by zolpidem binding affinity, are primarily restricted to alterations in α-subunit expression. Binding is not a specific measure of protein expression, however it may provide a limited description of protein combinations (different subunits associate as benzodiazepine receptor subtypes). The examination of these interactions may demonstrate how the $GABA_A$ receptor subunits *in vivo* perform with respect to benzodiazepine binding.

Benzodiazepine receptor subtypes (BZ) have been dissected with respect to their affinity for various concentrations of zolpidem (Figure 4.1). Due to limitations in availability of subtype specific ligands, pharmacologically we can only distinguish receptor subtypes based on α-subunit composition and those receptors' affinities for the benzodiazepine-like compound zolpidem. However, the fact that γ-subunit isoform switching may alter the benzodiazepine binding site recognition cannot be ignored, and in fact the replacement of β-subunit isoforms will alter the modulation of the receptor (Herb et al., 1992; Hadingham et al., 1993b; Wafford et al., 1993b; Ebert et al., 1994; Graham et al., 1996 and for review see Sieghart 1995). To include the possibility that an increase/decrease in specific binding of one or more subtypes may alter their percentage contribution to the total sites, the ratios between these BZ receptor subtypes after chronic treatment have been compared.

There were no significant changes in these ratios after either treatment regime. If the α-subunit mRNA steady state levels correspond to alterations in BZ receptor subtypes, there may be a slight increase in both BZ2 and BZ3 subtype binding. These results therefore stress the importance of the β- and γ-subunit present in the BZ receptor subtype; the examination of BZ subtypes cannot be made based on the isolated α subunit alterations.

It has been recently demonstrated that the β- and γ-subunits may alter zolpidem affinity, and when in combination with specific α-subunits the exact consequence of the association is unknown. For example, if the α5-subunit was in combination with the γ1-subunit, the decreased zolpidem affinity as a result of increased α5-subunit may be counteracted by the increased zolpidem sensitivity of γ1-subunit. Therefore rather than detecting an increase in the BZ 3 subtype, the increase in the α5-subunit was compensated for and subsequently the proportion of the BZ 3 subtype profile was normal. Another possibility for the disparity in BZ subtype changes may be that the time course for $GABA_A$ receptor mRNA alterations do not correlate to changes in protein expression, which subsequently alters the benzodiazepine subtype binding. Therefore examining protein products at the equivalent mRNA level time course may not verify protein alterations; moreover, the mRNA levels may not altogether be an appropriate measure to correlate with benzodiazepine binding characteristics.

The GABA enhancement of benzodiazepine binding is an apparent measure of coupling between the benzodiazepine modulation site and the GABA binding site. These results demonstrate that both diazepam dosing regimes significantly compromise this interaction compared to vehicle treated control cortical tissue. The uncoupling between sites may lead to decreased allosteric modulation; therefore, further diazepam treatment would fail to cause increased GABA binding, increased frequency of channel opening, and increased hyperpolarization of the neuron.

It is of interest to note that chronic diazepam treatment by daily injection (which has been shown previously to produce tolerance) results in a dramatic rise in α4-subunit mRNA levels, which in turn may lead to an increase in α4-subunit containing $GABA_A$ receptors. It is possible that an increase in these so called "diazepam insensitive" receptors may therefore play a role in the development of diazepam tolerance. However, chronic diazepam infusion has the opposite effect on α4-subunit mRNA levels, suggesting that the extent of tolerance development may be different between these dosing paradigms.

Diazepam insensitive receptor subtypes are the proportion of receptors which contain an α4- and or α6- subunit (Hadingham et al., 1996; Knoflach, 1996; Wafford et al., 1996; Whittemore et al., 1996). There was a significant difference between cortical tissue from diazepam injected and diazepam infused animals in total specific [^{3}H]-Ro 15-4513 binding, indicating that at a particular concentration, the number of sites has increased after daily injections. However, as demonstrated by the displacement of this ligand with diazepam, a larger percentage of the binding was diazepam insensitive, indicating an increase in α4/α6-subunit containing receptor subtypes in cortical tissue from diazepam injected animals relative to diazepam infused animals. This change in binding corresponded to an increase in the level of α4-subunit steady-state mRNA, and was consistent with the changes in steady-state mRNA levels that were observed.

A significant number of studies have examined the effects of chronic treatment on both $GABA_A$ receptor mRNA steady-state levels and $GABA_A$ receptor benzodiazepine binding characteristics, but results are difficult to compare. Experimental parameters have varied between studies: animal species, treatment regimes, doses, benzodiazepines, anatomical areas of the brain examined, methods for both quantification and analysis of alterations in gene expression (Heninger et al., 1990; Primus and Gallager 1992; O'Donovan et al., 1992a,b; Wu et al., 1994b; Zhao et al. 1994; Holt et al., 1996; Impagnatiello et al., 1996). Further, there have been varying methods used for benzodiazepine binding analysis and for measuring $GABA_A$ receptor coupling (Rosenberg and Chiu, 1981; Gallager et al., 1984a,b, 1985; Yu et al., 1988; Hernandez et al., 1989; Marley and Gallager, 1989; Tietz et al., 1989; Li et al, 1993; Wu et al, 1994a; Brett and Pratt, 1995). The differences in the dosing regimes range from oral guavage and intraperitoneal or depot injection to crystallized pellet or pump infusion, all which would result in different degrees of receptor occupancy. This study examined the question of chronic treatment, in the same animal species with the same daily dose, comparing only the

differences in mRNA levels and the subsequent benzodiazepine binding features in the rat cortex consequent to the use of two diazepam dosing paradigms.

This chapter demonstrates that comparative dosing paradigm is important to the induction of changes in $GABA_A$ receptor gene expression and in resultant benzodiazepine binding characteristics. Further, it is not only the absolute amount of drug at the receptor which leads to treatment dependent alterations in mRNA levels and benzodiazepine binding, but also the daily fluctuations in this drug level. While tolerance to injections of diazepam has been amply illustrated previously (for review see File and Pellow, 1990; Hutchinson et al., 1996), the question has never been extensively addressed when diazepam treatment occurs by infusion via osmotic minipumps.

Moreover, the increase in α4-subunit mRNA levels (shown after chronic daily injection) results in an increase in diazepam insensitive $GABA_A$ receptor subtypes, which may underlie the development of tolerance. It is possible that after chronic diazepam pump infusion, which exhibits opposite changes, tolerance would not be observed to the same extent. Tolerance to certain behavioral effects has been examined in mice with osmotic minipump infusion, but this has not been specifically correlated with $GABA_A$ receptor mRNA levels (Miller et al., 1988a, 1989). Luscombe and colleges (1994) have shown tolerance to the anxiolytic effects after 28 days with chronic chloradiazepoxide via osmotic minipumps in the rat, but again alterations in $GABA_A$ receptor gene expression were not examined. Gallager's group (Gallager et al, 1985; Gonsalves and Gallager 1987; Davis and Gallager 1988; Hernandez et al., 1989) did examine the development of anticonvulsant tolerance and an aspect of anxiolytic tolerance after chronic treatment with the silastic crystallized diazepam pellets; however, the kinetics of diazepam delivery were not explicitly determined and $GABA_A$ receptor subunit mRNA levels were not regularly examined.

In conclusion, the results of this chapter are interesting as they show differential alterations in $GABA_A$ receptor mRNA levels and resulting binding characteristics based on chronic diazepam treatment via two different dosing regimes, continuous pump infusion

and daily s.c. injection. It is possible that the differential changes in gene expression underlie the alterations in benzodiazepine binding, and are due to the different kinetics of drug administration. These results suggest that it is not only the level of diazepam occupation at the $GABA_A$ receptor that signals alterations in mRNA expression but the daily "withdrawal" or fluctuation in drug level. Further, if these changes in $GABA_A$ receptor mRNA level are related to the development of tolerance, then differences in the mRNA levels, and the subsequent alterations in protein level may correlate with differences in the development of behavioral tolerance which may occur between the two dosing regimes.

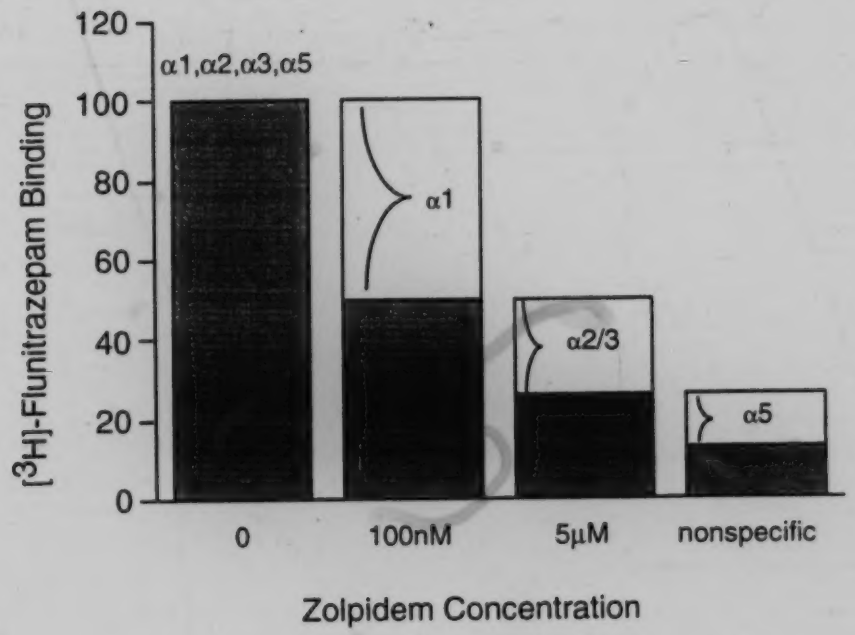


Figure 4.1:

This schematic bar graph represents the dissection of $GABA_A$ benzodiazepine (BZ) receptor subtypes, based on the particular α-subunit's affinity for the benzodiazepine site ligand zolpidem, which displaces the bound radioligand [^{3}H]-flunitrazepam. The α1-subunit isoform containing receptors (BZ1) have a 150 times greater affinity for zolpidem binding compared to α2/α3-subunit isoform composed $GABA_A$ receptors (BZ2). This can distinguish between what has been classified as BZ1 (α1-subunit containing) and BZ2 (α2- and α3-subunit containing) receptor subtypes. One can further separate a BZ3 $GABA_A$ receptor subtype into those receptors which contain primarily the α5-subunit isoform because of the extremely low affinity α5-subunit isoform containing receptors have for zolpidem.

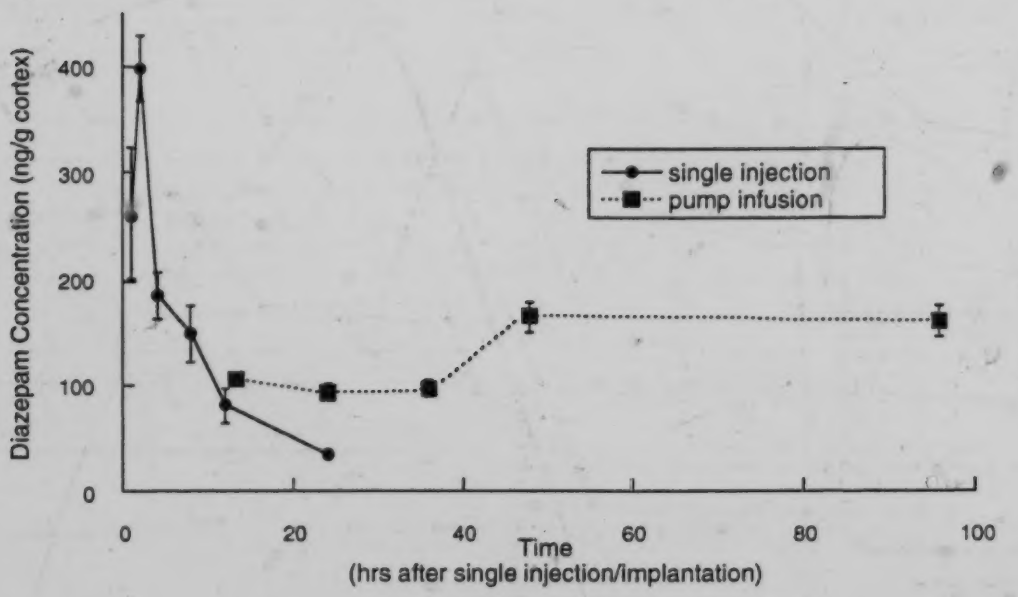


Figure 4.2:

The kinetics of diazepam concentration (ng/g cortex) in the rat measured by HPLC analysis. Each point represents the mean drug concentration for 2-3 animals sacrificed at a specific time point after either s.c. injection with 1.0 ml 15 mg/kg of diazepam (50% DMSO/50% PG vehicle), or after implantation of osmotic minipump delivering 15 mg/kg/day diazepam in the same 50% DMSO/50 % PG (v/v) vehicle. Error bars represent the SEM and the error bars for the 24 hour time point are within the symbol; n=3 for all time points with the exception of the 1 hour daily injected and the 36 hour pump infused time point where n=2.

Table 4.1:

Mean $GABA_A$ receptor α-, β-, γ-subunit mRNA steady-state levels as a percentage of their vehicle treated controls after 14 days chronic treatment with 15 mg/kg/day diazepam (in 50% DMSO/50% PG (v/v) via daily injection or pump infusion. Error is reported as the SEM calculated from 3-4 independent S1 nuclease assays performed on 4-6 animals per treatment group (n). $GABA_A$ receptor subunit mRNA measurements were normalized to those of the β-actin internal standard. * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$ using Student's unpaired *t*-test analysis within each subunit isoform comparing the diazepam treatment to vehicle control regime.

Significant differences in $GABA_A$ receptor mRNA levels between diazepam treatment and vehicle treated controls (values as a percentage of vehicle)		
Subunit Isoform	**Diazepam daily injection** (mean ± SEM, n)	**Vehicle daily injection** (mean ± SEM, n)
α1	96.1 ± 2.1 (13) *	100 ± 1.1 (36)
α3	108.0 ± 3.7 (19) **	100 ± 1.3 (35)
α4	117.9 ± 7.7 (14)**	100 ± 3.2 (39)
β2	91.6 ± 3.0 (12)	100 ± 2.5 (35)
β3	122.5 ± 6.9 (11)***	100 ± 2.8 (25)
Subunit Isoform	**Diazepam pump infusion** (mean ± SEM, n)	**Vehicle pump infusion** (mean ± SEM, n)
α4	79.6 ± 2.9 (16) ***	100 ± 4.8 (18)
α5	110.1 ± 2.0 (15)*	100 ± 1.7 (19)
β2	109.8 ± 3.5 (19)*	100 ± 3.8 (20)
γ1	133.3 ± 14.9 (16)**	100 ± 4.6 (25)
γ2	90.8 ± 3.7 (21)*	100 ± 3.4 (24)

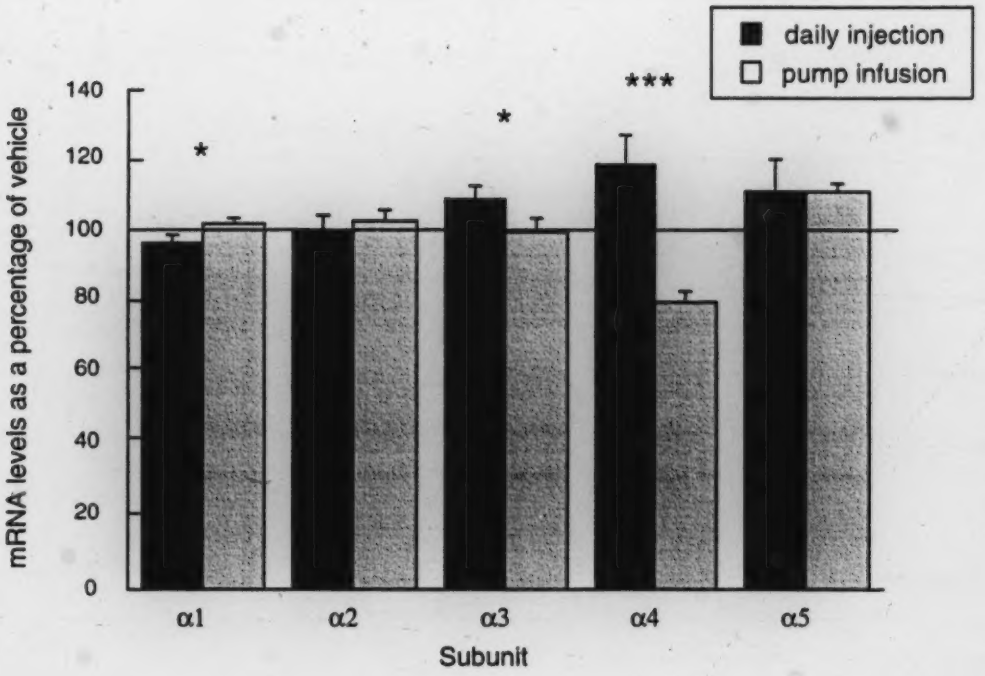


Figure 4. 3:

Mean $GABA_A$ receptor α-subunit mRNA steady-state levels as a percentage of vehicle treated controls after 14 days chronic treatment with 15 mg/kg/day diazepam (50% DMSO/50% PG vehicle) via daily injection (hatched) or pump infusion (gray). Error bars represent SEM calculated from 3-4 independent S1 nuclease assays performed on 4-6 animals per treatment group. $GABA_A$ receptor subunit mRNA measurements were normalized to those of the β-actin internal standard. * represents $P < 0.05$ and *** represents $P < 0.001$ using Student's unpaired *t*-test analysis within each subunit isoform comparing the two different treatment regimes.

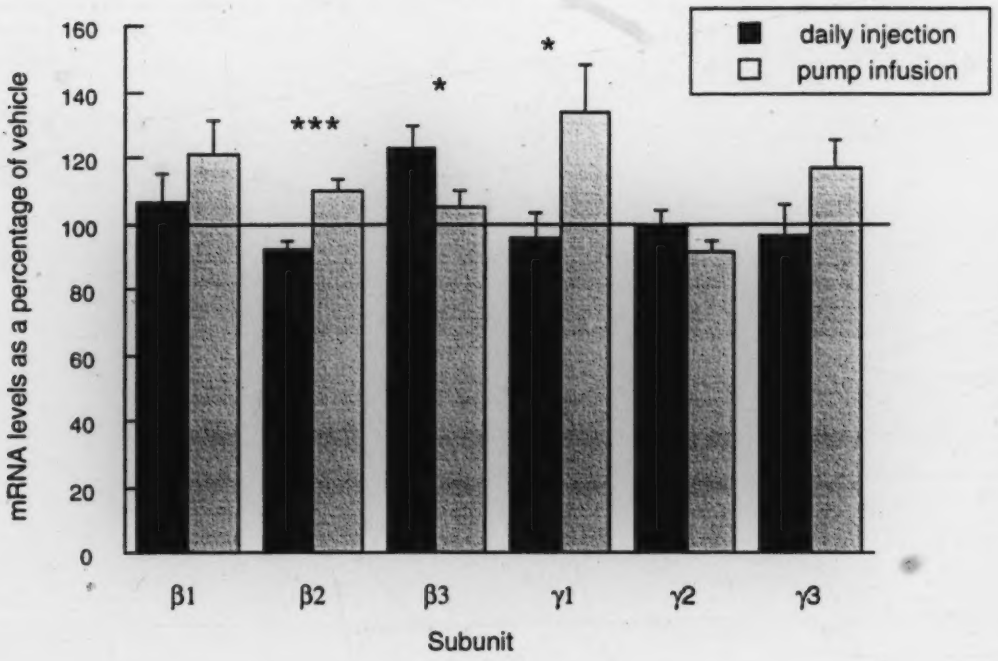


Figure 4.4:

Mean $GABA_A$ receptor β- and γ-subunit steady-state mRNA levels as a percentage of vehicle treated controls after 14 days chronic treatment with 15 mg/kg/day diazepam (50% DMSO/50% PG vehicle) via daily injection (hatched) or pump infusion (gray). Error bars represent SEM calculated from 3-4 independent S1 nuclease assays performed on 4-6 animals per treatment group. $GABA_A$ receptor subunit mRNA measurements were normalized to those of the β-actin internal standard. * represents $P < 0.05$ and *** represents $P < 0.001$ using Student's unpaired *t*-test analysis within each subunit isoform comparing the two different treatment regimes.

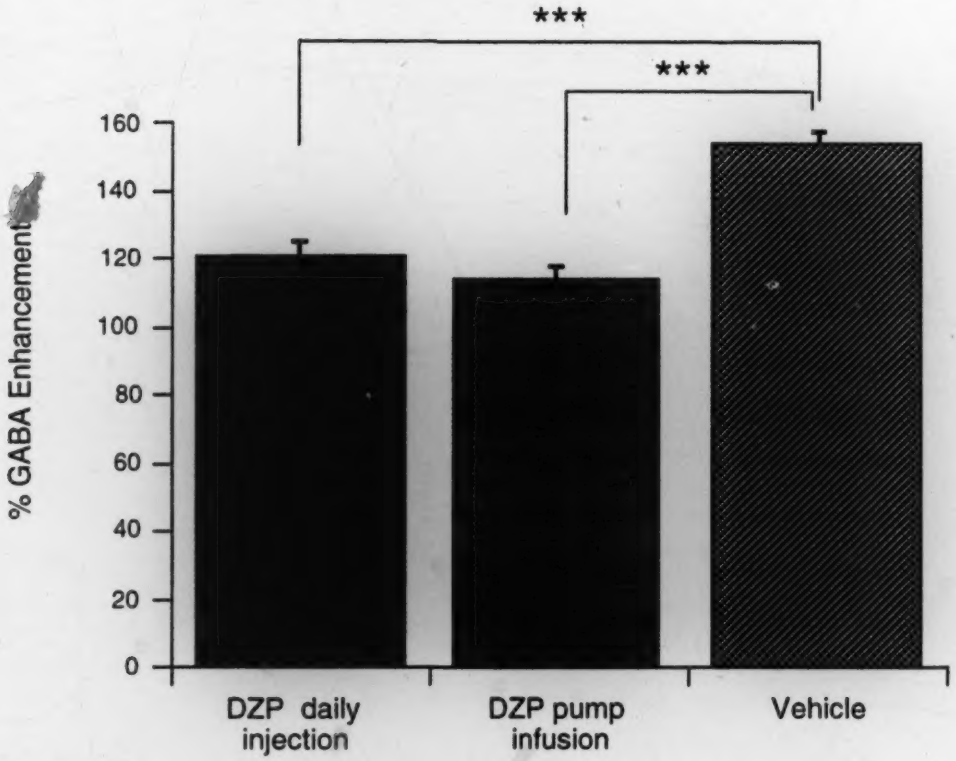


Figure 4.5:

The mean percentage of [^{3}H]-flunitrazepam binding enhancement after the addition of 100 μM GABA to cortical tissue from animals chronically treated with either 15 mg/kg/day diazepam via s.c. injection (hatched) or pump infusion (gray), or vehicle (50% DMSO/50% PG (v/v), black). Error bars represent SEM calculated from binding analysis completed in triplicate, with n=5 for daily injected, n=6 for pump infused, and n=8 for vehicle. *** represents $P<0.001$ using a one-way ANOVA followed by the Student Newman -Keuls post ANOVA test comparing the diazepam and vehicle treatment regimes.

	Benzodiazepine Subtypes			Benzodiazepine Subtype Ratios	
Treatment	BZ 1 (% of total)	BZ 2 (% of total)	BZ 3 (% of total)	BZ2/BZ1	BZ3/BZ2
daily s.c. injection	60 ± 0.4	32.2 ± 0.5	7.4 ± 0.3	0.533 ± 0.013	0.231 ± 0.011
pump infusion	58.4 ± 0.9	34.2 ± 0.7	7.4 ± 0.4	0.588 ± 0.02	0.216 ± 0.012
vehicle	60.1± 1.0	31.6 ± 0.4	8.3 ± 1.0	0.527 ± 0.011	0.262 ± 0.033

Table 4.2:

The mean percentage of BZ subtypes expressed relative to total [^{3}H]-flunitrazepam binding and the relative subtype ratios in cortical tissue from animals chronically treated with either 15 mg/kg/day diazepam via s.c. injection or pump infusion, or vehicle (50% DMSO/50% PG (v/v)). The binding assay was completed in triplicate and error represents SEM, with n=6 for daily injected and for pump infused, and n=4 for vehicle. A one-way ANOVA statistical analysis was performed and indicated no significant differences between treatment groups.

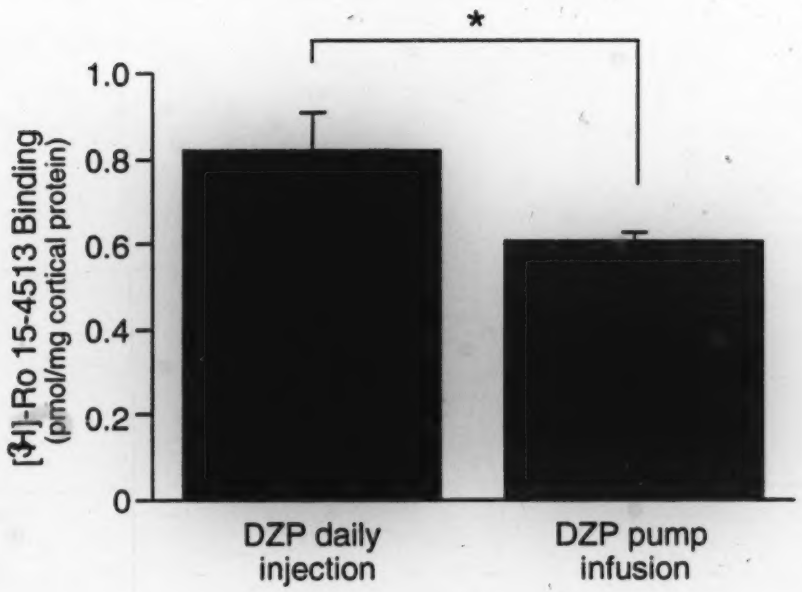


Figure 4.6:

The mean of total specific [^{3}H]-Ro 15-4513 binding (pmol/mg protein) in cortical tissue from animals chronically treated with either 15 mg/kg/day diazepam via s.c. injection (hatched) or pump infusion (gray). Nonspecific binding was defined using 10 μM cold Ro 15-4513. Error bars represent SEM calculated from binding analysis completed in triplicate, with n=6 for daily injected and for pump infused. * represents $P<0.05$ using Student's unpaired *t*-test. There was no difference in [^{3}H]-Ro 15-4513 binding between vehicle treatment groups (this combined mean vehicle total specific [^{3}H]-Ro 15-4513 binding was 7.04 ± 0.1 pmol/mg cortex, n=7).

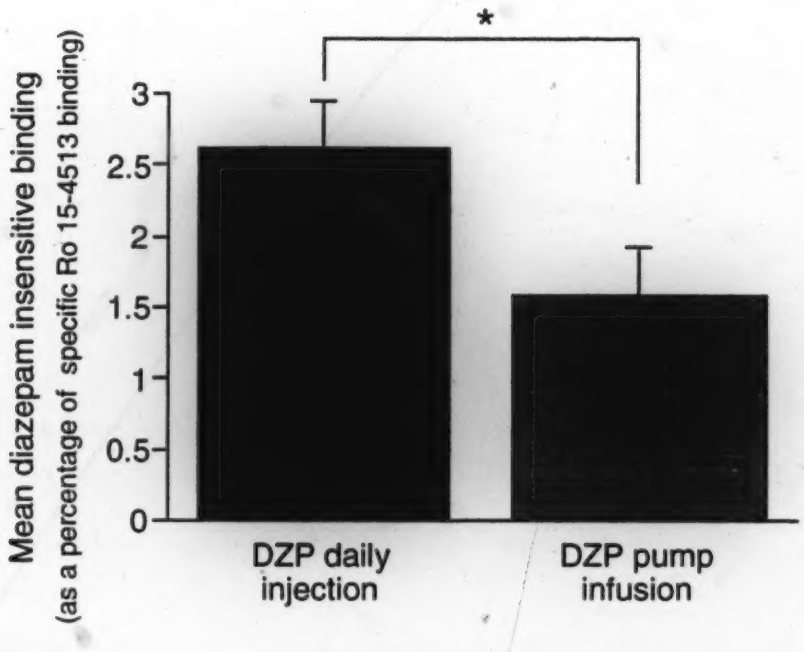


Figure 4.7:

The mean of proportion of cortical [^{3}H]-Ro 15-4513 binding after the addition of 10 μM diazepam (diazepam insensitive binding) from animals chronically treated with either 15 mg/kg/day diazepam via s.c. injection (hatched) or pump infusion (gray). This portion is relative to the total specific [^{3}H]-Ro 15-4513 binding within the treatment group. Error bars represent SEM calculated from binding analysis done in triplicate, with n=6 for daily injected and n=5 for pump infused. * represents $P<0.05$ using Student's unpaired *t*-test. There was no difference in [^{3}H]-Ro 15-4513 binding between vehicle treatment groups (the combined mean vehicle DIS proportion was 2.48 ± 0.48%, n=7).

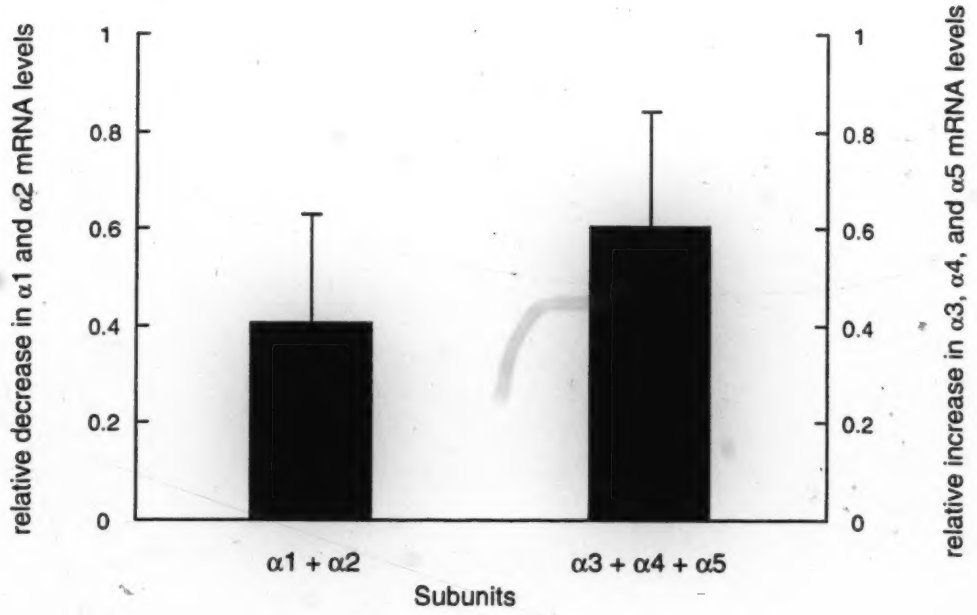


Figure 4.8:

$GABA_A$ receptor steady-state mRNA levels from daily injected animals, as a percentage of vehicle controls, were normalized with respect to their relative abundance in cortex. The mean absolute changes (an overall decrease for α1-subunit mRNA levels, an overall increase for α3- and α4-subunit mRNA levels) were compared using Student's unpaired *t*-test analysis. There was no significant difference between the magnitude of the decrease in the α1- and α2-subunit mRNA levels and the magnitude of the sum increases in the α3-, α4 and α5-subunit mRNA levels.

CHAPTER 5

Behavioral and Anticonvulsant Tolerance Following Chronic Diazepam Administration by Daily Injection or Continuous Infusion

INTRODUCTION

Diazepam has sedative, anxiolytic, muscle relaxant and anticonvulsant properties (see Chapter 1), but the use of this compound is limited by the development of tolerance to particular aspects of its pharmacological profile and this has become a major problem in prescribing this drug (Tyrer, 1987; Roy-Byrne, 1991). Dependence may be depicted as an alteration in the physiological state caused by changes or adaptations in response to a drug challenge; one measure of this manifestation is the development of tolerance to behavioral and anticonvulsant effects of diazepam administration (Woods et al., 1987a; File 1985; File and Pellow, 1990; Loscher et al., 1996a,b). Two distinct models of functional tolerance, decremental and oppositional, may be applied to predict adaptive processes that are initiated following drug treatment (Littleton and Harper 1990). Loss of drug effectiveness within the system is considered a decremental adaptation. In contrast, oppositional tolerance develops as a counteractive adaptation within the system to oppose drug induced effects. However, one must consider that in the development of tolerance decremental and oppositional adaptive models may be involved dependently or concurrently. The molecular mechanisms which underlie tolerance to benzodiazepines are not understood.

Diazepam's interaction with a specific recognition site on the $GABA_A$ receptor in the mammalian CNS results in allosteric modulation of the effects of GABA and, therefore, allows manipulation of inhibitory neurotransmission (see Chapter 1). We have shown that two diazepam treatment regimes differentially alter the expression of $GABA_A$ receptor genes, and the subsequent binding characteristics of the receptor (discussed in Chapter 4). Briefly, exposure of rats to the same daily dose of diazepam (15 mg/kg) delivered for 14 days either by single daily injection or by constant osmotic minipump infusion, produced significantly different changes in specific $GABA_A$ receptor subunit mRNA levels and subsequently altered $GABA_A$ receptor benzodiazepine binding modulation. For instance,

there was a significant increase in cortical steady state α4 mRNA levels of rats receiving daily subcutaneous diazepam injections relative to cortical α4 steady-state mRNA levels in animals receiving diazepam via continuous infusion. Further, although both cortical membrane preparations similarly show compromised GABA enhancement of flunitrazepam binding, membrane preparations from daily injected animals had a significantly increased diazepam insensitive benzodiazepine binding component.

If changes in $GABA_A$ receptor gene expression are related to the development of tolerance, the different dosing regimes of equivalent daily diazepam may result in the differential development of behavioral and anticonvulsant tolerance. However, it is not only the level of diazepam occupation at the $GABA_A$ receptor which signals these alterations, but that these alterations may be dependent upon the fluctuations in the drug level. Therefore in this study we compared the development of tolerance to chronic diazepam treatment, given by two different dosing regimes, daily injection and continuous infusion, which result in equivalent daily doses. Specific behavioral measurements of anxiety, sedation and exploration (using elevated plus maze, social interaction and holeboard), and measurements of seizure protection to pentylenetetrazole (PTZ) challenges have demonstrated differential adaptations to chronic diazepam treatment.

[2] PART 1

Development of Anticonvulsant Tolerance

[2] This work was conducted with the help of Dr Chien-Tsai Lai.

Materials and Methods

Drug Treatment

Male Sprague Dawley rats (250-300g) were acclimatized prior to drug therapy as described in Chapter 3. Surgical implantation of osmotic minipumps delivering 15 mg/kg/day diazepam (Chapter 3) occurred 48 hours or 14 days prior to the PTZ convulsant challenge. Similarly, animals receiving diazepam via daily s.c. injection received either 2 or 14 injections of diazepam, the last dose being administered 2 hours before the PTZ challenge.

Pentylenetetrazole Challenge

Rats were challenged via intraperitoneal injection of 70 mg/kg pentylenetetrazole (PTZ) dissolved in saline. (The PTZ dose was explored prior to initiation of the experiment; sample animals were tested to establish a non-lethal yet, convulsive dose within 3 minutes of PTZ administration.) Experimental animals were immediately placed in activity cages and monitored; scoring took place in the initial 6 minutes following the injection and the degree of convulsive activity was ranked by a blinded scorer within this period. Surviving rats were then sacrificed within one hour of the PTZ challenge by decapitation. The ranking of seizure activity was roughly based on examples from the literature (Zang et al., 1997).

Stage 0: no apparent effect, animals appears normal

Stage 1: head nodding, panting, freezing and crouching

Stage 2: hyperactive with tonic convulsions

Stage 3: tonic convulsions and animal involuntarily jumps back

Stage 4: tonic convulsions and animal falling on back or side

Stage 5: tonic-clonic convulsions and animal falling on back or side

Stage 6: tonic-clonic convulsion followed by death

Statistical Analysis

Rankings within a treatment group were analyzed via the non-parametric Kruskal-Wallis test followed by a Mann-Whitney U test comparing acute, chronic and vehicle treatment within each dosing regime. The vehicle groups from day 2 and day 14 were not different than each other (Mann-Whitney U test) and were therefore combined. A two-way ANOVA was applied between the two treatment regimes at the two time points to establish if treatment had the same effect at all time points or if one treatment regime was more effective than the other in convulsant protection.

Results

After 2 and 14 day treatment with daily s.c. injection of 15 mg/kg diazepam the animals were significantly ($P<0.01$) protected relative to vehicle treated animals. The mean rankings were 0.78 ± 0.15 (9) (mean $\pm$ SEM; n) after 2 days treatment, 1.50 ± 0.22 (10) after 14 days and 5.65 ± 0.13 (20) after vehicle treatment. However, the acute 2 day treatment of diazepam was significantly more effective compared to the chronic 14 days diazepam treatment ($P=0.05$; Figure 5.1A).

Following 2 and 14 day diazepam treatment with the same daily dose of 15 mg/kg administered via continuous infusion, animals were similarly protected relative to their vehicle controls. After vehicle treatment the mean ranking was 5.72 ± 0.18 (18), which was significantly higher than the ranking after 2 days treatment of 2.14 ± 0.46 (7), and after 14 days diazepam treatment, 3.71 ± 0.81 (7). There were no significant differences between the acute and chronic pump treatment (Figure 5.1B).

Further, two-way ANOVA analysis established there was a significant difference between daily injection and pump treatment ($F[1, 28] =0.82$). This indicates that the daily injection schedule was more effective at seizure protection relative to the same daily dose

administered via continuous infusion. Moreover, there was a significant difference between the time course in both treatment regimes ($F[1, 28] = 6.42$); however, there was no significant interaction and therefore no differential development of tolerance between treatment regimes.

Discussion

The 70 mg/kg i.p. PTZ challenge was a sufficient dose to elicit convulsions with both vehicle treatment groups, the mean ranking above 5.5, indicating that with the exception of one pump vehicle animal, all vehicle animals had full clonic tonic seizures, however, not all seizures were fatal. The mechanism of action of PTZ on the $GABA_A$ receptor has been reported to be complex. It appears to act on the GABA-site, the benzodiazepine-site as well as the ion channel (Maksay et al., 1994). The PTZ seizure model is often employed for the examination of benzodiazepine ligands (Rundfeldt et al., 1996; Loscher et al, 1996a,b). Previous studies have used bicuculline as the convulsant administered via tail vein infusion (Gonsalves and Gallager, 1987; Hernandes et al., 1989; Impagnatiello et al., 1996; Zanotti et al., 1996), and PTZ has similarly been administered (Rosenberg, 1995); however, the relatively simple technique of a bolus challenge was utilized (Doble et al., 1993; Ramsey-Williams et al, 1994; Chen et al., 1996). Following PTZ administration, animals were monitored for differences in the degree of protection between treatment times and diazepam dosing regimes.

Statistical analysis revealed that all time courses and treatment regimes provided significant seizure protection relative to the vehicle controls. However, statistically the only indication of tolerance was the significant difference between the acute and chronic daily injection of diazepam, demonstrating that after 14 days of diazepam, treatment was not as effective at seizure protection. This tolerance was similar to that reported in the literature

(Impagnatiello et al., 1996; Zanotti et al., 1996). Although there was a trend for a reduction in diazepam efficacy between acute and chronic osmotic minipump infusion with diazepam, the difference did not reach significance. It has been reported that diazepam given by continuous infusion with silastic pellets produces tolerance after 14 and 21 days to the convulsants bicuculline and PTZ (Gonsalves and Gallager, 1987; Hernandes et al., 1989; Ramsey-Williams et al., 1994; Rosenberg 1995). The discrepancy between these studies may be due to either the different drug levels at the time of the challenge or the convulsant model chosen for each study; in mice it has been demonstrated that tolerance characteristics are dependent on the seizure model (Loscher et al., 1996a). However, no adequate comparisons have evaluated the development of tolerance between different treatment regimes of the same drug.

Analysis by two-way ANOVA indicated that the daily diazepam injection regime was more effective than the continuous infusion and that both acute treatments were more proficient than the corresponding chronic regimes. The data also demonstrate the same pattern of protection between the treatment regimes; all doses were protective compared to vehicle controls but both chronic regimes were less potent than the corresponding acute treatments, indicating that both treatment regimes may demonstrate a trend towards tolerance production. This was supported by the two-way ANOVA, which demonstrated no significant interaction between the treatment regimes and the time course, and therefore no differential development of tolerance. Differences in the degree of scatter or variance are apparent between dosing regimes, with a larger range of scatter in the pump treated animal seizure rankings (Figure 5.1b). It may be that animal heterogeneity to the convulsant response increased with lower levels of diazepam, and this individual variability was masked by higher more effective diazepam levels in the injection treated animals. Studies in mice have previously demonstrated a certain degree of strain differences in response to PTZ challenges (File et al., 1985). Nonetheless, it may also be that pump infusion results in more highly variable, or inconsistent protection.

The most apparent difference between dosing regimes when analyzing the ranked data was the difference in baseline between the daily injection and the pump infusion. The lower baseline found with the injection regime may be due to higher diazepam drug levels at the time of the PTZ challenge. As seen previously in Chapter 4 (Figure 4.1), the maximum brain diazepam level occurs 2 hours post injection, which would be approximately 4 times the level of diazepam in the brain of pump treated animals. This difference in drug level may be the reason why daily injection was more effective in protection and may be why tolerance cannot be distinctly observed when comparing between the two paradigms. Further, the increased variation and higher baseline in the pump infused animal group may be obscuring the detection of distinct diazepam anticonvulsant tolerance.

To explore the potential differences in tolerance development to the anticonvulsant effects of diazepam administered via two dosing regimes, it would be beneficial to perform the PTZ convulsant challenge at a time point when brain levels of diazepam were equivalent. For instance, at 4-8 hours after the diazepam daily injection, drug levels would be equivalent to those produced by pump infusion (Figure 4.1). The results of such an experiment may give more information about the development of tolerance between dosing regimes and the relative effectiveness of diazepam provided via daily injection or continuous infusion.

[3]PART 2

Development of Sedative and Anxiolytic Tolerance

[3] This work was conducted in the Department of Psychopharmacology at Guy's Hospital, London, U.K. in the laboratory of Dr. Sandra File. Dr. Cathy Fernandes' experienced aid and advice and Elaine Irvine's help were greatly appreciated during this study. The behavioral tests were scored by trained laboratory personnel.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, UK), weighing approximately 150-180g, were housed in groups of 5 or 6 so that each cage contained at least one animal from each time course; 5 days prior to testing the rats were separated and housed individually. Animals were housed in the same animal room, maintained at 22 °C, with lights (<50 scotopic lux) on from 0700-1900h. Food and water were freely available. The experimental procedures carried out in this study were in compliance with the UK Animal Scientific Procedures Act 1986 (Home Office Project License Number: 90/00656).

Drug Treatment

The procedure was similar to that previously described for chronic administration via osmotic minipumps (Alzet®, Palo Alto, CA), and the preparation and surgical implantation of the osmotic minipumps delivering 15 mg/kg/day diazepam was carried out as previously described (Chapter 3; Tanay et al., 1996). As before, prior to surgery rats were pretreated with a 0.5 ml subcutaneous injection of 2 mg/kg flumazenil, to reduce mortality during and directly following surgery. Rats were anaesthetized by isoflurane (Abbott, UK) inhalation and osmotic minipumps were subcutaneously implanted in the dorsal thoracic area. Animals were monitored daily and the pumps were turned every second day. Another group of rats received daily morning s.c. injections of either 15 mg/kg diazepam dissolved in 50% DMSO/50% PG (v/v) or vehicle alone. A low injection volume (less than 0.3 ml) was used to minimize the amount of vehicle injected. Flumazenil was a generous gift from Hoffmann-La Roche, Basel, Switzerland.

Quantification of drug levels by reversed-phase HPLC

Diazepam levels were quantified as previously described (Chapter 3).

Behavioral Tests

Holeboard

The holeboard was a wooden box 60 x 60 x 35 cm with 4 holes, each 6.5 cm in diameter, equally spaced in the floor. Each rat was placed in the center of the holeboard for a 5 minute trial and at the end of each trial any fecal boluses were removed and the box was wiped clean with a damp cloth. The number of head dips and the time spent head-dipping were measured by the interruption of infrared beams from cells located immediately beneath the edges of the holes. Locomotor activity and rearing were measured by the interruption of infrared beams from cells located in the walls of the box, 4.5 and 12.5 cm, respectively, from the floor. The holeboard apparatus provides independent measures of motor activity and exploration (File and Wardill, 1975a,b; File, 1991).

Social interaction test

The social interaction test arena was a wooden box 60 cm square x 35 cm deep. Infrared photocells were mounted in the walls of the box, 4.5 and 12.5 cm from the floor and the interruption of these beams provided automated measures of locomotor activity and rearing, respectively. A closed circuit camera was mounted vertically above the arena and the rats were observed from a monitor in an adjacent room. Photocell output and the scores of the observer were entered into a computer; details of this test are described previously (File 1980 and 1993). The light level in the test room was < 50 scotopic lux, for the testing sessions.

Rats were transferred 5 days before testing to single housing and one day prior to testing, animals were allocated to test partners within their drug group, on the basis of weight, such that members of a pair did not differ by more than 10-20 g (n=8 pairs/group). (Both members of a test pair received the same chronic treatment.) At the beginning of each social interaction trial, members of a test pair were placed at opposite corners of the arena, facing each other. An observer blind to the drug treatment of the rat pairs recorded the time each pair spent in active social interaction. (Individual rat scores were not taken,

as the behavior of one rat was not considered to be independent of its partner's behavior). The following social interaction behaviors were scored, combined and expressed in seconds for each rat pair: sniffing, following, grooming, crawling under or over a partner, boxing, wrestling, kicking, biting, mounting and horizontal submission. (Incidences of passive interaction, when the rats were in contact but not interacting with each other were also scored). At the end of each social interaction test, any fecal boluses were removed from the arena which was then wiped clean with a damp cloth.

Elevated plus-maze

The elevated plus-maze was made of wood and had two open arms (50 x 10 cm) and two enclosed arms of the same size with walls 40 cm high, elevated 50 cm above the ground. A camera was mounted vertically above the maze and the behavior was scored from a monitor in an adjacent room. Each rat was placed in the central square (10 x 10 cm), facing an enclosed arm, and allowed to freely explore the maze for 5 min. At the end of each trial, any fecal boluses were removed from the maze which was wiped clean with a damp cloth. The numbers of entries onto, and the times spent on, open and closed arms were recorded by an observer blind to the drug treatment. The number of entries onto, and time spent on, the distal part (last 25 cm) of the open arms was also recorded. Four paws into, and two paws out of, an arm defined an arm entry and exit, respectively. The percentage time spent on the open arms [open time/(open + closed time) x 100] was calculated, as was the percentage number of open arm entries. An increase in the percentage of time spent on the open arms is interpreted as an anxiolytic response, whereas the number of entries into closed arms provides a measure of general activity (Pellow *et al.*, 1985; File, 1992). In addition, the time spent on the central square was also measured. The influence of the central square on the behaviors detected in the elevated plus-maze is largely unknown, although it has been suggested that the exploratory behaviors seen in this area of the maze may relate to some kind of assessment and/or decision-making process (Trullas *et al.*, 1991; Rodgers *et al.*, 1992; Cruz *et al.*, 1994; Rodgers and Johnson, 1995).

Procedure

One hundred and forty rats were randomly allocated to either s.c. or pump infusion treatment groups (see Table 5.1). Within each treatment group, animals were randomly allocated to the following groups (n=16 rats/group): control, 7, 14 and 28 days diazepam. In order to equate handling and injection experience and in order to test all the s.c. injected rats on the same day, these groups of rats received 28 days of daily s.c. injections with vehicle or diazepam, as appropriate. Rats implanted with osmotic minipumps were regularly handled both prior to, and following, pump implantation. An additional acute diazepam group was added to both treatment groups, with one group receiving 27 days s.c. injection of vehicle followed by a single s.c. injection of diazepam (n=8); the other acute group received a diazepam filled pump 26 hours prior to testing (n=4). All animals were weighed every 3 days and vehicle control animals from both treatment regime groups were treated identically to their diazepam treated cage mate. Rats in the s.c. daily injection treatment groups were tested 2 hours after receiving the s.c. injection of vehicle or diazepam, as appropriate.

To test for the development of tolerance to diazepam, pairs of rats were tested on day 28 for 4.5 minutes in the social interaction test under low light, unfamiliar conditions and the time each pair spent in active social interaction and passive interaction was recorded. Immediately after the end of the social interaction test, the pair of rats were separated and one rat was tested for 5 minutes in the holeboard and the other rat was tested for 5 minutes in the elevated plus-maze. All testing took place under quiet conditions and low light (<50 scotopic lux). The development of tolerance was assessed by comparing vehicle, acute, 7, 14 and 28 days of diazepam treatment.

Animals were killed immediately after testing by decapitation and the brains immediately removed, dissected, frozen in a 95% (v/v) ethanol/dry ice bath and stored at -80 °C. All behavioral testing and sacrificing took place between 0900 - 1330h.

Statistics

The effects the two different treatment paradigms (pump infusion versus daily s.c. injection administration) on the development of tolerance to the sedative and anxiolytic effects of diazepam were assessed by two-way ANOVA, with length of diazepam treatment as one factor **(time)** and treatment paradigm as the other factor **(treatment)**. A significant **time** factor would indicate that tolerance to the behavioral effects of diazepam had developed and a significant **treatment** factor would indicate a significant behavioral difference between the two treatment paradigms. A significant **treatment x time** interaction would indicate that the time course of behavioral tolerance to diazepam had been influenced by the treatment paradigm.

Differences between individual groups in the holeboard and social interaction tests were assessed by one-way ANOVA, followed by Duncan's multiple range tests; it is these statistical tests which are reported in the tables and figures. The differences between individual groups in the elevated plus-maze test were assessed by Kruskal-Wallis followed by Mann-Whitney U tests. It is these statistical differences which are shown in the tables and figures.

Results

Drug Levels

Diazepam levels in the cortex, quantified by HPLC analysis, were measured from randomly chosen animals chronically treated with 15 mg/kg/day diazepam via daily s.c. injection or continuous pump infusion (Table 5.2). Diazepam levels 2-3 hours following diazepam injection were 477.9±17.8 ng/g (mean ± SEM) after a single acute injection, and following 7, 14 and 28 days of daily injection were 593.5±28.4 ng/g, 808.7±40.0 ng/g and 766.9±41.8 ng/g, respectively. The mean diazepam level after 26 hours of acute

infusion was 109.0±22.6 ng/g; further, after 7, 14 and 28 days continuous infusion the diazepam levels were 133.5±14.2 ng/g, 153.3±19.8 ng/g and 237.0±15.5 ng/g, respectively.

Holeboard Test

There was a significant decrease ($P<0.01$) in the motor activity of acute diazepam injected animals relative to vehicle, 7, 14 and 28 day injection treatment groups (Figure 5.2). The number of beam breaks decreased after a single injection to 39.0±21.6 from 264.3±20.9 beam breaks recorded by the vehicle group, and 207.8±46.1, 297.6±45.8, and 248.3±27.5 beam breaks following chronic treatment for 7, 14 and 28 day respectively. Further, there was a significant decrease in the number of animal rears during this test comparing acute injection (2.7±2.0) to vehicle (18.5±1.3; $P<0.01$), 7 (10.0±2.5; $P<0.05$), 14 (13.5±2.6; $P<0.01$) and 28 (11.8±2.5; $P<0.01$) days daily injection. There was also a significant difference ($P<0.05$) between the vehicle and 7 day treatment group (Figure 5.3). The number of head dips decreased in acutely treated injection animals relative to the other groups in this dosing regime. After a single diazepam injection animals decreased the number of head dips from 12.4±2.3 ($P<0.05$) to 4.3±1.5; these measurements were again returned to previous values after 7 days treatment, with the number of head dips returning to 10.3±2.1, and at 14 and 28 days treatment with daily diazepam injection the number of head dips were 13.9±2.2 and 13.6±2.1, respectively (Figure 5.4). There was a significant difference between vehicle ($P<0.05$), 14 ($P<0.05$) and 28 ($P<0.01$) days relative to acute treatment with respect to the time the animal spent head dipping. The acute animal group spent 4.4±1.8s head dipping whereas vehicle, 14 and 28 day groups spent 12.1±2.9s, 13.5±1.3s and 16.1±3.2s respectively. (Figure 5.5).

After continuous infusion with diazepam there was no significant difference between treatment groups in the motor activity and number of rears, measured by the number of beam breaks, but in the acutely infused animals, measures of sedation were reduced; however, this reduction did not reach significance (Figure 5.2 and 5.3).

However, the number of head dips and the time spent head dipping by the acutely infused animals relative to the vehicle and chronic groups was significantly decreased ($P<0.01$; Figure 5.4 and 5.5). The number of head dips dropped to 2.7±.7 after 26 hours exposure to diazepam, relative to 13.3±2.4 by the vehicle group, 14.8±1.1 after 7 days, 15.0±1.9 after 14 days and 13.3±1.8 after 28 days treatment. The amount of time the animal's head spent in the holes decreased with acute diazepam infusion to 1.5±0.8s relative to 11.5±2.9s spent by the vehicle group ($P<0.05$), 13.7±2s, 13.5±1.8s by the 7 and 14 day treatment groups ($P<0.01$) and 12.5±3.3s spent by the 28 day chronic infused group ($P<0.05$).

The analysis of treatment regimes by two-way ANOVA demonstrated a significant effect of treatment time ($F[4, 58]= 8.39$) on motor activity, indicating that with both dosing regimes the acutely treated animals were significantly compromised. There was no significant interaction between the two treatment paradigms (Figure 5.2 and 5.3). This statistical test established that the measurement of rears was significantly different ($F[4, 58]= 4.80$) in the time course of treatment, with the acute group demonstrating fewer rears. There was also a significant treatment difference ($F[1, 58]= 8.80$) between the dosing regimes, where the daily injection treatment group overall had fewer rears. Two-way ANOVA further confirmed the difference between the acute treatment and other groups in the number and time of head dips ($F[4, 58]= 9.18$, and $F[4, 58]= 6.20$, respectively), but there was no difference between the different regimes. There were no treatment X time interactions in the holeboard measurements.

The acute treatment groups for either dosing regime were not included in the social interaction test or elevated plus-maze tests as acute sedative effects of diazepam seriously confound detection of any changes in anxiety in these tests.

Social Interaction

After chronic daily diazepam injections for 7, 14 and 28 days there were no significant differences between the animals receiving drug and their vehicle control group in regard to motor activity and rearing (Figure 5.6 and 5.7). However there was a significant

decrease in the time which animal pairs spent in social interaction after 28 days, relative to both the vehicle and 7 day treatment groups ($P<0.05$; Figure 5.8). The amount of time vehicle pairs spent interacting was 90.2±13.4s and after 7 days of chronic diazepam treatment the amount of time was 88.8±10.8s, which was significantly greater than the amount of time 28 day treatment pairs spent in interaction (52.3±7.4s). There were no significant differences in the amount of time animals spent in passive interaction. Following chronic pump infusion with the same daily dose of diazepam, there were no significant differences between the treatment and vehicle groups in any of the social interaction or motor activity measures (Figures 5.6, 5.7, and 5.8).

Analysis of the social interaction test by two-way ANOVA indicated that motor activity measurements were not significantly different in the time course or treatment regimes. There was a significant difference ($F[1, 53]= 11.28$) between the treatment regimes when comparing the number of rears, with daily injected animals rearing less than the pump infused animals. Similar to the one-way ANOVA within each regime, there was no significant difference in the number of rears between the vehicle controls and the chronically treated animals. There was a significant difference between treatment regimes in the time spent in total interaction ($F[1, 53]= 37.17$), where the daily injected animals were less interactive than the pump infused animals. There was no significant difference with the time course of treatment, but unlike the other social interaction measurements, there was a significant treatment X time interaction in the amount of time animals spent in total social interaction ($F[3, 53]= 2.71$).

Elevated Plus Maze

The elevated plus maze test for anxiety reports a number of anxiolytic parameters. There was no significant difference in the number of closed arm entries following daily injection with diazepam compared to the vehicle controls, indicating that these animals were not sedated (Figure 5.9). There was a significant difference ($P<0.01$ and $P=0.001$) between the number of open arm entries, the percent number of these entries and the

percent of time spent in the open arms between the vehicle control groups and the 7, 14 and 28 day injection treatment groups. The number of open arm entries increased from 0.38±0.26 (vehicle control) to 3.9±0.5 after 7 days, 3.6±0.6 after 14 days and 2.4±0.43 after 28 days treatment (Figure 5.10). The percent number of entries onto the open arm further demonstrated an increase after chronic diazepam injection treatment from 2.3±1.5% vehicle entries to 20.4±2.5%, 19.8±3.2%, and 15.0±2.5% following 7, 14 and 28 days dosing, respectively (Figure 5.11). The percentage of time spent on the open arms by animals receiving chronic treatment also increased significantly relative to the vehicle control group (1.2±0.8%; Figure 5.12) after 7 (18.3±3.0%), 14 (17.2±2.3%), and 28 (13.4±3.5%) days treatment. The number of entries on to the distal portion and the time spent in this area illustrated a different pattern of significance. There was a significant increase ($P<0.01$) after 7 (2.0±0.5) and 14 (1.9±0.6) days treatment relative to vehicle (0.1±0.1) in the number of distal arm entries (Figure 5.13); however only 7 days treatment resulted in a significant increase ($P<0.05$) in the amount of time spent on this distal portion compared to the vehicle control group (14.6±4.9s versus 0.04±0.04s; Figure 5.14). There was no significant difference in the amount of time spent in the central portion of the plus maze after chronic daily diazepam injections (Figure 5.15).

Continuous infusion of diazepam for an extended period did not alter the number of closed arm entries by animals in the elevated plus maze (Figure 5.9). However the number/percent number of open arm entries, percent time in the open arms, and number of entries and the time spent in the distal portion of the arms were significantly elevated after 7 days continuous diazepam infusion relative to the vehicle, 14 and 28 day treatment groups ($P<0.05$ and $P<0.01$). The number of open arm entries increased from a baseline of 0.1±0.1 to 2.4±0.7 after 7 days and returned to 0.5±0.3 by 14 days treatment (Figure 5.10). The percent number entries increased from the vehicle level of 0.8±0.8% to 16.0±4.6% after 7 days and quickly returned to 3.6±1.9% and 2.8±1.8% by 14 and 28 days respectively (Figure 5.11). The percentage of time spent in the open arms was

0.6±0.6% in the vehicle control group and this significantly increased to 17.0±4.0% after 7 days, which was significantly different to 2.6±1.3% and 0.2±0.1% of time after 14 and 28 days continuous infusion (Figure 5.12). Chronic 7 day diazepam pump treatment increased the number of entries onto the distal arm to 1.8±0.6 and the time spent on this distal portion to 12.4±3.6s. These values are significantly elevated relative to the vehicle control and 28 day groups which did not enter the distal portion at all during testing (Figure 5.13 and 5.14). After 14 days treatment the number of entries onto and the time spent on the distal part of the arm were 0.1±0.1s and 0.6±0.6s respectively, which were significantly different than their respective values at 7 days diazepam infusion. Interestingly, there was a significant change in the amount of time pump treated animals spent on the central square during the test. The vehicle control group spent 111.9±9.2s, which was a significantly less amount of time than at 7 (154.8±8.9s; $P<0.01$), 14 (141.5±25.5s; $P<0.05$), and 28 (151.7±11.0; $P<0.01$) days treatment (Figure 5.16).

Analysis of this test with two-way ANOVA confirmed the previous analysis and illustrated a number of treatment X time interactions. In the number and the percent number of open arm entries parameters there were significant differences ($F[3, 51]= 15.73$) within the time course of treatment and between the treatment regimes ($F[1, 51]= 32.48$). Further, there was an interaction ($F[3, 51]= 3.81$) in each measure, demonstrating a differential development of change with respect to the treatment regimes and the time course for these changes (Figure 5.10 and 5.11). The above pattern of change was identical to the percentage of time spent on the open arms, with the exception that the treatment X time interaction was of greater significance ($F[3, 51]= 4.89$; Figure 5.12). There was a significant difference in the time course of treatment ($F[3, 51]= 5.18$ for time distal and $F[3, 51]= 9.88$ for number distal entries) and between the different regimes ($F[1, 51]= 6.39$ and $F[1, 51]= 8.71$); however, there was no significant interaction (Figure 5.13 and 5.14) in the distal arm measurements. With regard to the time spent in the central square by the test animals, there was a significant difference between the two dosing regimes ($F[1,$

51]= 23.52), and furthermore there was a significant interaction (F[3, 51]= 3.25) between the time course and the treatment paradigms (Figure 5.15).

Discussion

The cortical diazepam levels following both daily injection and pump infusion for various time points were similar to what was expected according to the acute kinetic experiments previously performed (Figure 4.1). The drug levels after chronic daily injection were measured 2-3 hours post injection and therefore reflect the peak concentration and accumulation of drug at these time points. These daily injection levels were approximately 7-8 times higher than diazepam levels measured 24 hours after the last injection, and after 28 days treatment, the brain levels are about 5% of daily administered diazepam dose. The mean drug levels following diazepam pump infusion demonstrate an accumulation of drug that was not previously detected (Chapter 3). However the levels were still similar to what may be expected after this treatment regime and the brain levels were approximately 2% of the daily dose administered (Table 5.2). The drug levels were quantified to ensure that animals received drug and that the levels were relatively consistent within a treatment group. There were different levels of cortical diazepam quantified between the daily injected and pump infused animals at different time points, and although this may be a possible mechanism for the differences in behavior demonstrated in this study, previous work has found that drug levels may not correlate with behavioral alterations (File et al., 1985; Fernandes et al., 1996).

The results of the holeboard test indicate that although acutely pump infused animals were not as sedate as the acutely injected animals, overall the diazepam regime results in acute sedation, and tolerance developed to this parameter by treatment day 7 of both treatment regimes. This observation was in accordance with the reports from the

literature (File et al., 1985; File and Fernandes, 1996). The acute pump infused animals showed a greater variability in motor activity, which may be a result of individual animal heterogeneity or may be due to the variability in drug delivery. If pump surgery played a role in the measure of sedation in acutely infused animals, one would expect these animals to be more sedate than the injected animals, and this was not the case. The daily injected animals demonstrated a more marked reduction in motor activity, and, with respect to the number of rears, the s.c. injected group did not recover to pre-treatment vehicle levels. This difference in rears may be the result of ataxic muscle relaxant effects of the acute diazepam bolus injection, and may not be a reflection of sedation. Sedative effects measured via the social interaction test indicate similar results to the holeboard test with respect to vehicle and chronic diazepam treatment in animal motor activity and the number of rears. Further, animals from the daily injected treatment groups demonstrated a significant decrease in the overall number of rears, which was correspondingly seen in the holeboard test. It is important to remember that although the social interaction test scores are the results from animal pairs (receiving the same treatment and time schedule), it reflects the same pattern of sedation demonstrated by the holeboard test. Furthermore, the deficit in sedation after chronic 7, 14 and 28 day treatment with both dosing regimes was further shown in the elevated plus maze test by the lack of alterations in the number of closed arm entries.

Tolerance to the acute effects of diazepam treatment on the number and time spent head dipping occurred with both treatment regimes, demonstrating a similar decrease in exploration which returned to baseline with a time-dependent development of tolerance. These exploration/sedation parameters in combination with the previous sedation parameters of the holeboard test indicate that the exploration of these animals was equally affected by diazepam chronic treatment and appears to be dissociated from changes in locomotor activity. Overall this test indicates that the daily injected animals are generally more sedated than pump treated animals, however tolerance develops to the chronic

sedative effects following both treatment regimes; furthermore, the exploration of these animals following diazepam treatment is not regime dependent.

The social interaction test also gives an measure of anxiety and accordingly benzodiazepines have been shown to increase the amount of time animals spend interacting, and this increase demonstrates the anxiolytic effects of the benzodiazepines (for review see File 1992; Treit and Menard, 1998). Further, it has been demonstrated that administration of anxiogenic agents results in a decrease in the time spent in social interaction (File and Pellow, 1983; File, 1984; 1985; 93; File et al., 1991) which is similar to that which is seen during benzodiazepine withdrawal (File et al., 1991). The different diazepam dosing schedules resulted in significant differences in the amount of time animals spent in social interaction. After chronic diazepam daily injection there was a decrease in the time spent in total interaction which reached significance by day 28 relative to both vehicle and 7 day treatment, indicating there was an increase in anxiety in animals which received 15 mg/kg/day chronic diazepam administration for 28 days via daily injection. After the same daily dose and time course of treatment there were no differences within the pump infused animal groups. Further, analysis indicated that differences are both treatment and time related, where daily injections resulted in a decrease in social interaction by day 28 which was not observed in the pump infusion groups. Therefore this social interaction test indicated that chronic diazepam daily injection was anxiogenic and this was not seen with chronic diazepam infusion. The anxiogenic effect of chronic daily injections may be the result of oppositional tolerance, and because the doses of diazepam administered were high and the fluctuations of drug level at the receptor would be great perhaps the receptor system over-adapted and the increase in anxiety detected may be a type of reverse tolerance. Another possible explanation was the development of acute withdrawal, where the daily injections trigger an "off" mechanism at the receptor due the massive and rapid activation/saturation of the $GABA_A$ receptor by diazepam. Therefore 2 hours after the injection, the drug levels dropped and caused acute withdrawal.

The anxiolytic measurements from analysis of the elevated plus maze indicate quite different results than the social interaction test, thereby demonstrating that these two anxiolytic tests measure different "types" of anxiety as previously described with factor analysis (File, 1992). It may be perhaps that the social interaction test was more sensitive to the "on/off" effects at the $GABA_A$ receptor. Animals receiving diazepam via daily injections demonstrated very little anxiety at all time points tested, but by 28 days of chronic treatment partial tolerance to the anxiolytic effects had developed. This partial tolerance was demonstrated by the difference in the number of open arm entries between 7 and 28 days treatment, and by the lack/loss of significance between the vehicle and 28 day treatment groups in the distal arm measurements. Pump treated animals were anxiolytic following 7 days treatment, but tolerance to this diazepam affect was significant by day 14 and maintained at day 28 following continuous diazepam infusion. There were significant interactions between treatment and time, indicating that the development of anxiolytic tolerance was affected by the dosing regime. Pump infused animals developed tolerance to anxiolytic effects at a faster rate than animals which were injected daily; however, the lack of treatment X time interaction in the distal arm measurements, which may be a more sensitive measure of anxiety, indicates that in this measure tolerance developed equally with both treatment paradigms.

It is of further interest to note that diazepam pump treated animals spent more time in the central square relative to their vehicle controls and to the diazepam daily injected treatment groups. There was also a significant interaction between treatment regime and time, where daily injected animals with extended treatment showed a decreased in the amount of time spent in the central square which is directly opposite to the continuously infused animals. The exact meaning of this measurement is not agreed upon, but there is some evidence that it may be an indication of impulsiveness and decision making (Trullas et al., 1991; Rodgers et al., 1992; Cruz et al., 1994; Rodgers and Johnson, 1995). With

regards to the present study, the exact nature of the measurement is an aside, but the two equivalent daily dose paradigms demonstrate another difference in behavioral effect.

The results presented in this chapter demonstrate that there was similar tolerance development to sedation and a differential development of tolerance to anxiolytic effects after chronic diazepam treatment with the same daily dose, administered either via daily s.c. injection or continuous infusion. Different mechanisms of tolerance have been implicated; however, in combination with the previous chapter this is the only instance where biochemical parameters can be extrapolated to behavioral measures of tolerance. By directly correlating the molecular and biochemical changes identified in Chapter 4 to the behavioral changes seen in this study, it may reveal the relationship between the $GABA_A$ receptor composition/function and behavioral consequences. For instance both treatment regimes demonstrated similar tolerance development to sedative effects on motor activity and exploration, and both treatment paradigms had a similar increase in the $GABA_A$ receptor α5-subunit mRNA levels (however this change did reach statistically significant) and demonstrated a compromised GABA enhancement of flunitrazepam binding. There was a significant difference in the development of anxiolytic tolerance. Anxiogenic effects may correlate to the increase in $GABA_A$ receptor α4-subunit gene expression and the diazepam insensitive binding component, which subsequently occurs after 14 days of daily diazepam injections. This biochemical alteration may be responsible for the loss or inverse diazepam efficacy demonstrated in the social interaction test.

This study further emphasizes that the $GABA_A$ receptor system appears to be able to detect modulation differences. $GABA_A$ receptor "self" regulation was demonstrated with equivalent daily diazepam, administered by two distinct dosing regimes, resulting in differential biochemical and behavioral alterations. Variation in the continuous or fluctuating levels of receptor occupancy differentially alters gene expression, which subsequently adjusts the functional properties of the receptor and how it may be modulated.

These differences in the formation of novel/different GABAergic connections may result in the formation of unique animal behavior.

Treatment group	Days			
Daily Injection	1-14	15-21	21-27	28 (TEST DAY)
Vehicle	VEHICLE	VEHICLE	VEHICLE	VEHICLE
Acute	VEHICLE	VEHICLE	VEHICLE	DIAZEPAM
7 day	VEHICLE	VEHICLE	DIAZEPAM	DIAZEPAM
14 day	VEHICLE	DIAZEPAM	DIAZEPAM	DIAZEPAM
28 day	DIAZEPAM	DIAZEPAM	DIAZEPAM	DIAZEPAM
Pump Infusion	1-14	15-21	21-27	28 (TEST DAY)
Vehicle	VEHICLE	VEHICLE	VEHICLE	VEHICLE
Acute				DIAZEPAM
7 day			DIAZEPAM	DIAZEPAM
14 day		DIAZEPAM	DIAZEPAM	DIAZEPAM
28 day	DIAZEPAM	DIAZEPAM	DIAZEPAM	DIAZEPAM

Table 5.1:

The treatment groups were randomly determined before initiation of treatment. In the injection group acute animals received 27 days vehicle injection prior to administration. Pump implanted animals had their pumps turned every second day and all animals were equivalently handled and weighed every 3 days. All animals within a treatment regime were tested on the same day. Diazepam (15 mg/kg/day) was dissolved in 50% DMSO/50% PG (v/v) vehicle, and injection volumes were limited to less than 300 µl.

Diazepam Cortical Drug Levels (ng/g cortex) (mean ± SEM; n)		
Treatment Period	**Daily Injection**	**Pump Infusion**
Acute	477.9 ± 17.8 (8)	109.0 ± 22.6 (4)
7 days	593.5 ± 28.4 (13)	133.5 ± 14.2 (13)
14 days	808.7 ± 40.0 (12)	153.5 ± 19.8 (15)
28 days	766.9 ± 41.8 (15)	237.0 ± 15.5 (15)

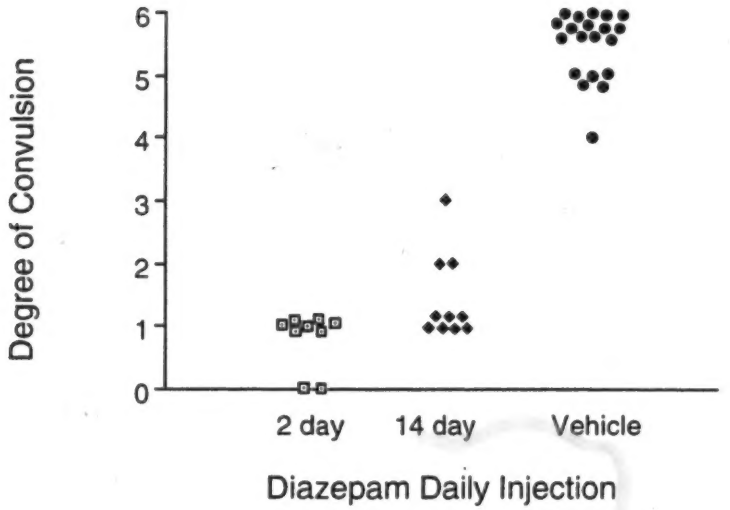
Table 5.2:

The diazepam concentrations (ng/g cortex) in the rats were measured by HPLC analysis. Each value represents the mean drug concentration after either s.c. injection with 15 mg/kg/day of diazepam (50% DMSO/50% PG (v/v) vehicle), or after implantation of osmotic minipump delivering 15 mg/kg/day diazepam in the same 50% DMSO/50% PG (v/v) vehicle. Animals were sacrificed immediately following behavioral testing; for daily injected animals this was 2-3 hours post injection. Error bars represent the SEM and n values range from 4-15.

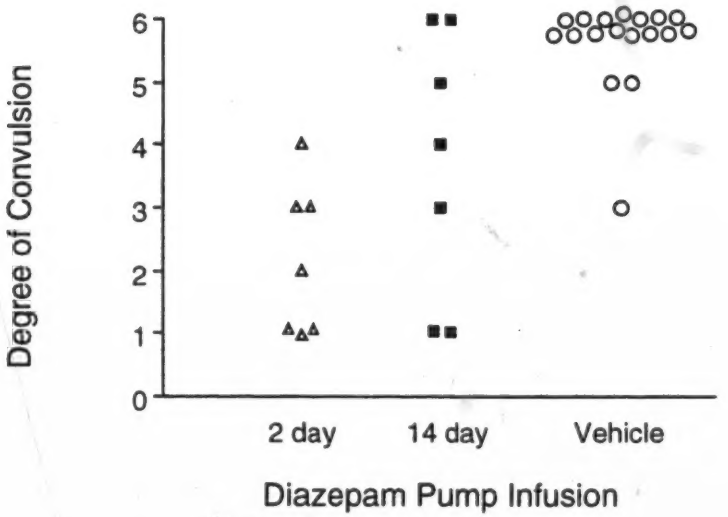
Figure 5.1:

The degree of animal convulsion was ranked immediately following PTZ challenge. Each point in the scatter plot represents a test animal. Vehicle rankings for both treatment regimes were above 5.5, indicating the effectiveness of the PTZ challenge. Following acute diazepam injection the mean seizure ranking value was 0.78±0.15 (9) (mean±SEM; n) and following 14 days of diazepam injections the mean seizure ranking was 1.40±0.22 (10) (Figure 5.1a). These values were significantly different from their vehicle controls ($P<0.01$) and were significantly different compared to each other ($P=0.05$). Following acute diazepam infusion the mean seizure ranking value was 2.14±0.46 (7), and after 14 days this value was 3.71±0.81 (7). Both these values were significantly different from their vehicle controls ($P<0.05$; Figure 5.1b). Statistical analysis performed was a Kruskal-Wallis followed by a Mann Whitney-U test. * represents $P<0.05$, ** represents $P<0.01$.

A.


Degree of Convulsion
6
5
4
3
2
1
0
2 day
14 day
Vehicle
Diazepam Daily Injection

B.


Degree of Convulsion
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5
4
3
2
1
0
2 day
14 day
Vehicle
Diazepam Pump Infusion

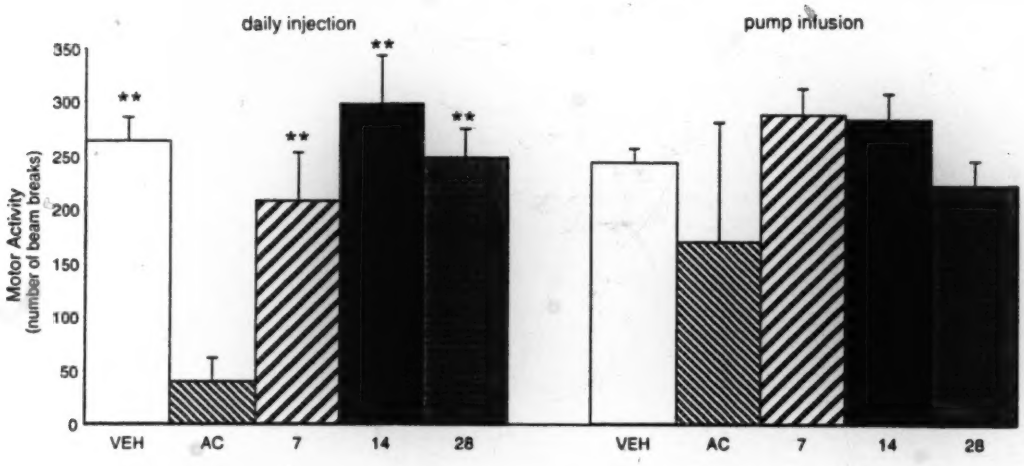


Figure 5.2:

Motor activity of individual animals was recorded as the number of beam breaks within the holeboard arena. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle and 28 day treatment groups). For the pump infusion group mean values were determined from 6-8 test animals, with the exception that there was n=3 in the acute group. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test. ** represents a $P<0.01$ relative to the acute treatment group.

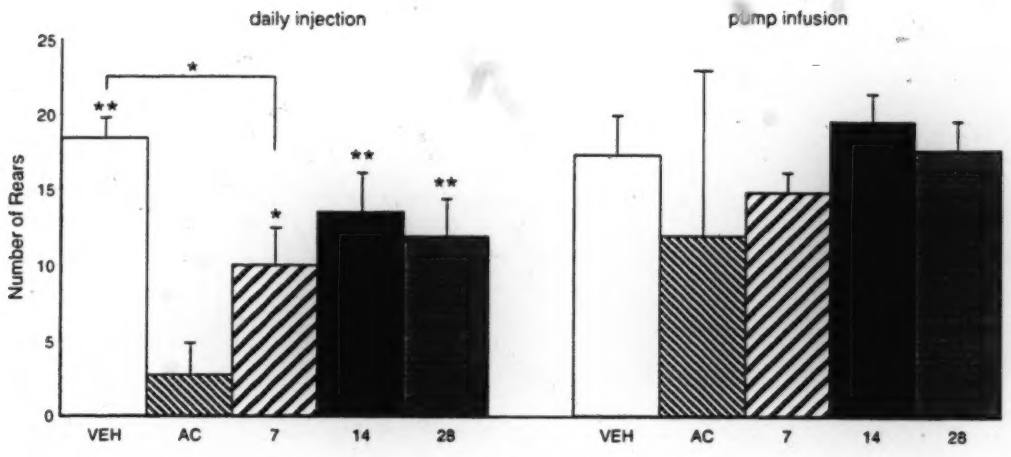


Figure 5.3:

The number of animal rears for individual rats was recorded as the number of beam breaks within the holeboard arena. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle and 28 day treatment groups). For the pump infusion group mean values were determined from 6-8 test animals, with the exception that there was n=3 in the acute group. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test. * represents a $P<0.05$ and ** represents a $P<0.01$, relative to the acute treatment group. However the vehicle injection group was significantly different from the 7 day treatment group.

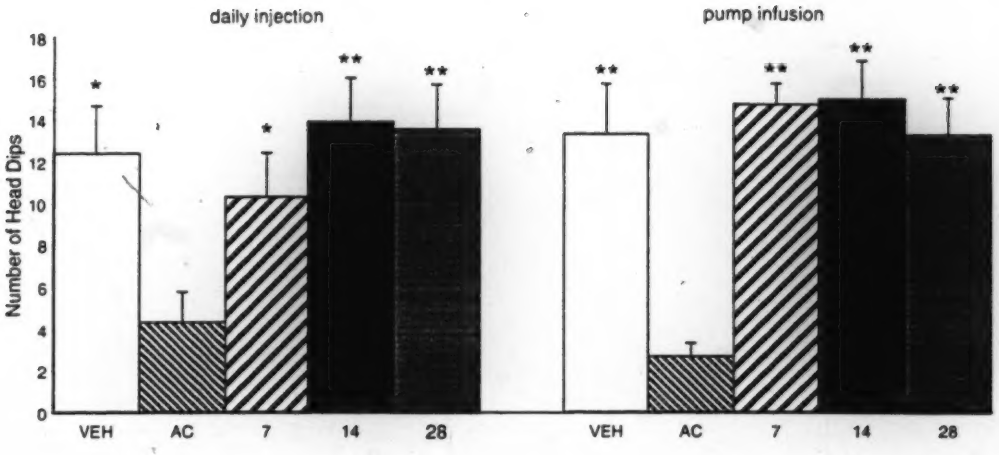


Figure 5.4:

The number of head dips for individual rats was recorded as the number of beam breaks within the holes of the floor of the holeboard arena. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle and 28 day treatment groups). For the pump infusion group mean values were determined from 6-8 test animals, with the exception that there was n=3 in the acute group. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test. * represents a $P<0.05$ and ** represents a $P<0.01$, relative to their respective acute treatment group.

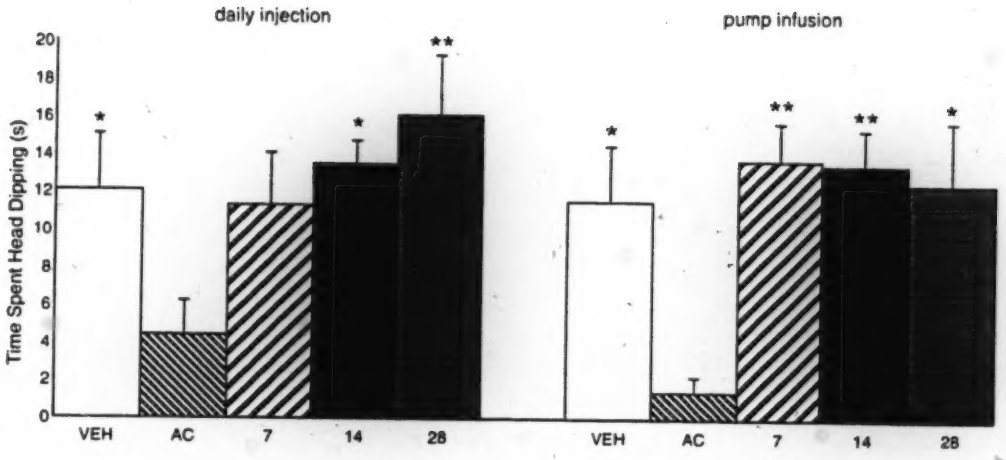


Figure 5.5:

The amount of time individual rats spent with their head down the holes in the holeboard arena. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle and 28 day treatment groups). For the pump infusion group mean values were determined from 6-8 test animals, with the exception that there was n=3 in the acute group. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test. * represents a $P<0.05$ and ** represents a $P<0.01$, relative to their respective acute treatment group.

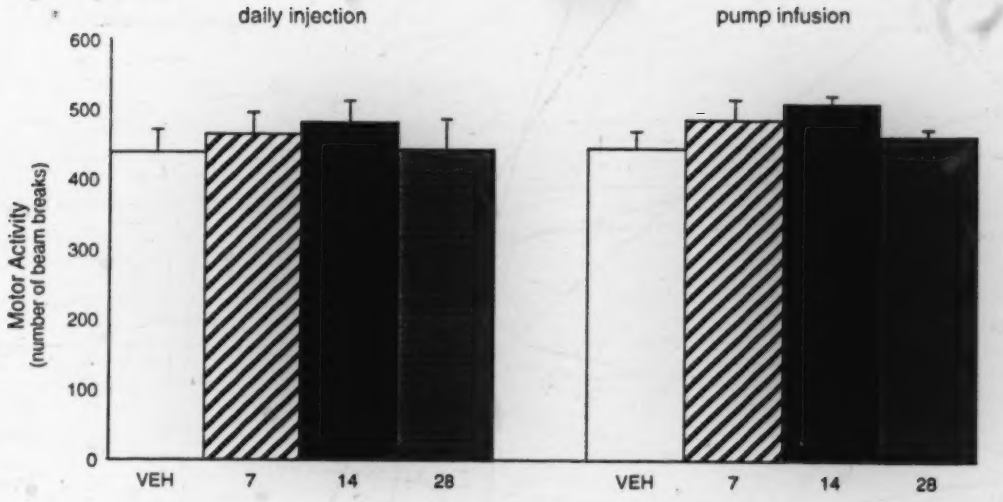


Figure 5.6:

Motor activity for animal pairs was recorded as the number of beam breaks within the social interaction test area. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=7 for vehicle treatment group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle treatment group). Acute treatment groups were not included in analysis of this test as sedation may affect animal's interaction. Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test.

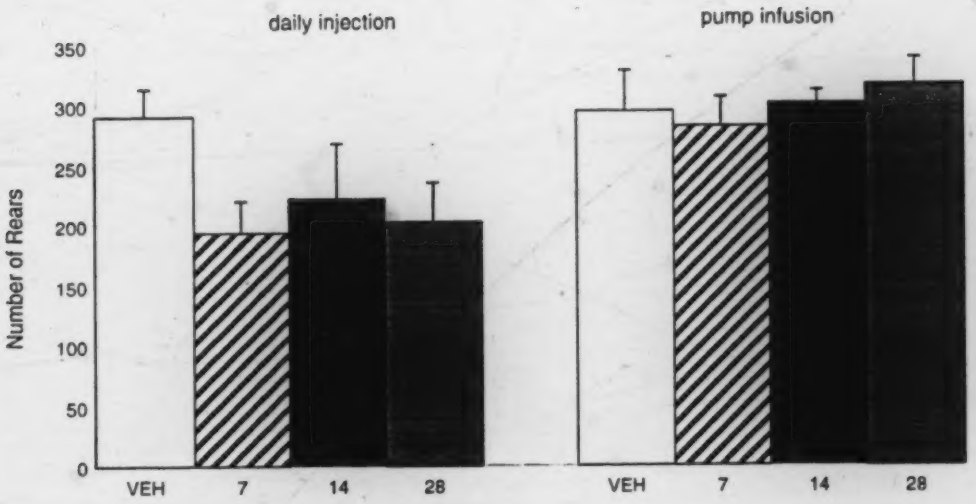


Figure 5.7:

The number of animal rears for rat pairs was recorded as the number of beam breaks within the social interaction test area. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=7 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test.

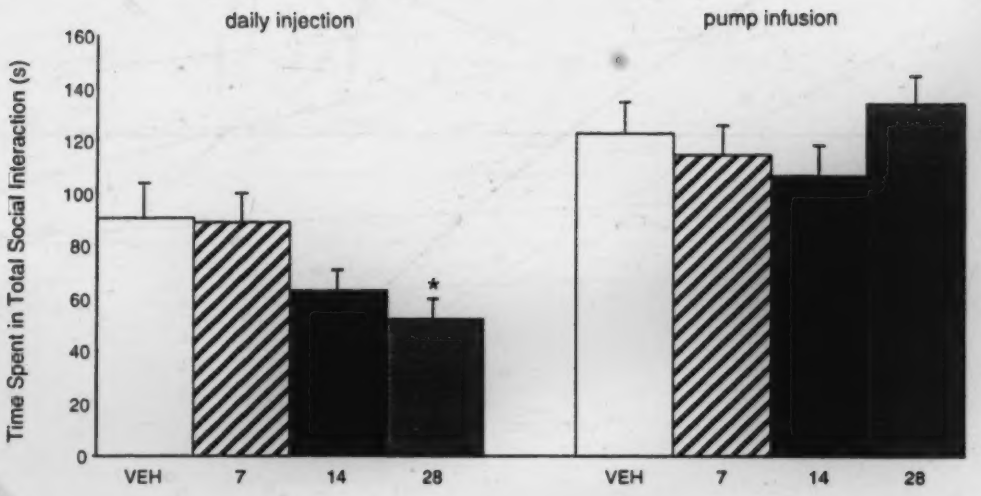


Figure 5.8:

The time spent in total social interaction for each rat pair. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test pairs (n=7 for vehicle group). For the pump infusion group mean values were determined from 6-8 test pairs (n=6 for vehicle group). Statistical analysis was performed using a one-way ANOVA, followed by Duncan's multiple range test. * represents $P<0.05$, where the injection 28 day treatment group was different relative to the injection vehicle and 7 day groups.

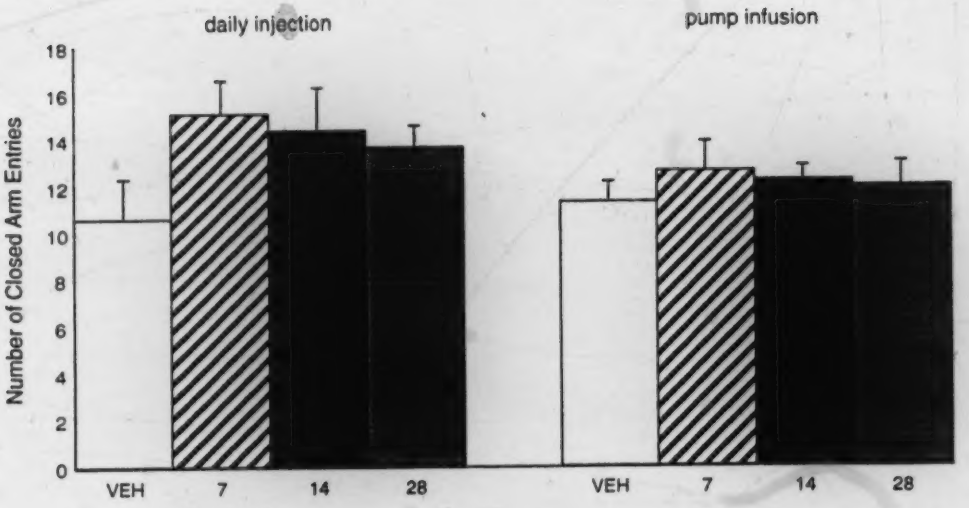


Figure 5.9:

The number of closed arm entries on the elevated plus-maze. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group).

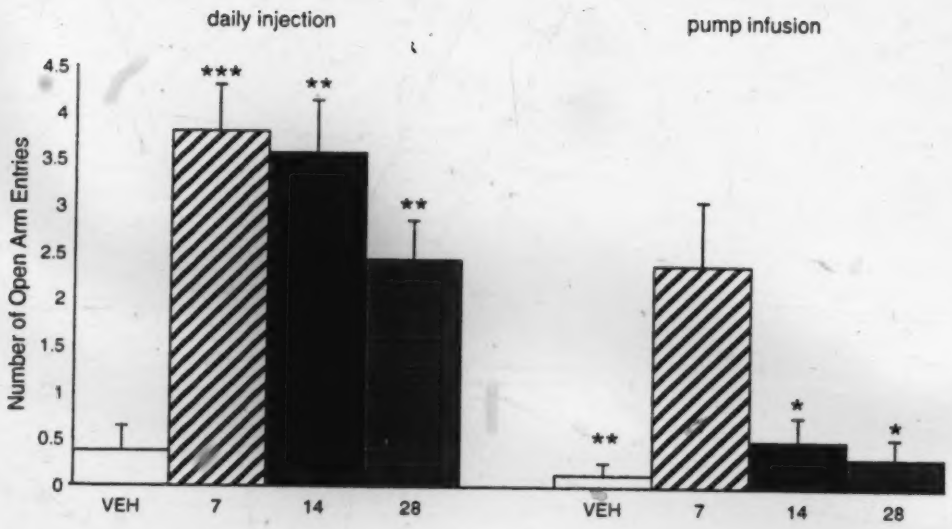


Figure 5.10:

The number of open arm entries on the elevated plus-maze. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. * represent $P<0.05$, ** represents $P<0.01$, and *** represents $P=0.001$. The daily injection vehicle was significantly different relative to the 7, 14 and 28 day diazepam injection groups. For the pump infusion group the differences were relative to the 7 day treatment group.

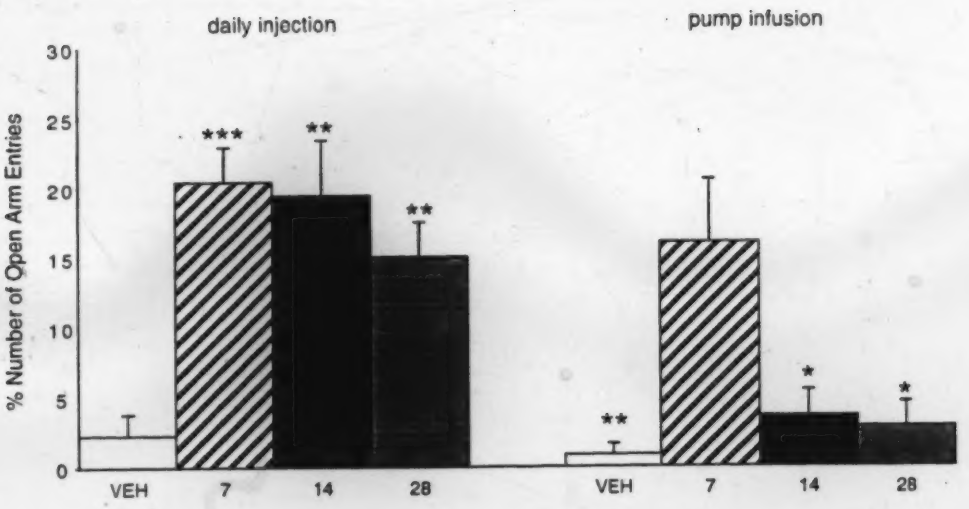


Figure 5.11:

The percentage number of open arm entries on the elevated plus-maze was calculated as [number of open arm entries/(number open + number closed) x 100]. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P=0.001$, relative to the vehicle injection group and for the pump infusion group the differences are relative to the 7 day diazepam infusion group.

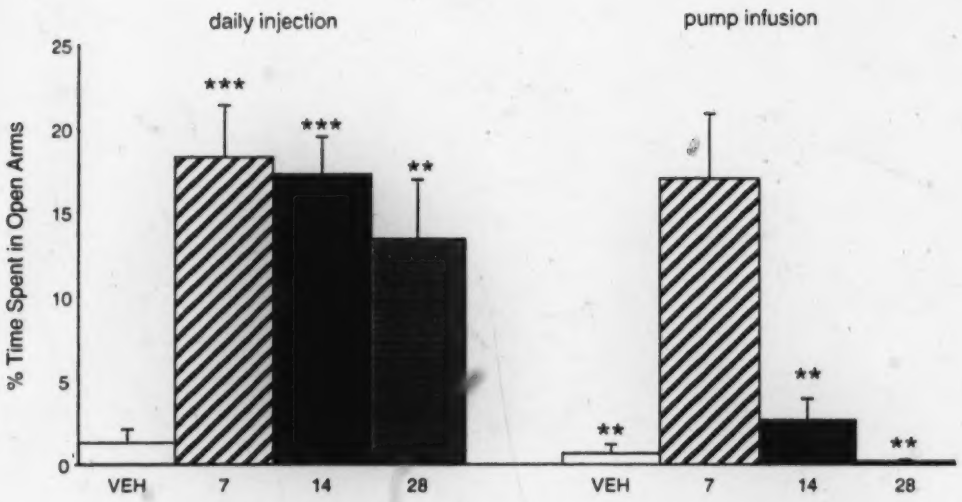


Figure 5.12:

The percentage of time spent in the open arms was calculated as [open time/(open + closed time) x 100]. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. ** represents $P<0.01$ and *** represents $P=0.001$, relative to the vehicle controls in the injection paradigm and for the pump infusion group the differences are relative to the 7 day diazepam treatment.

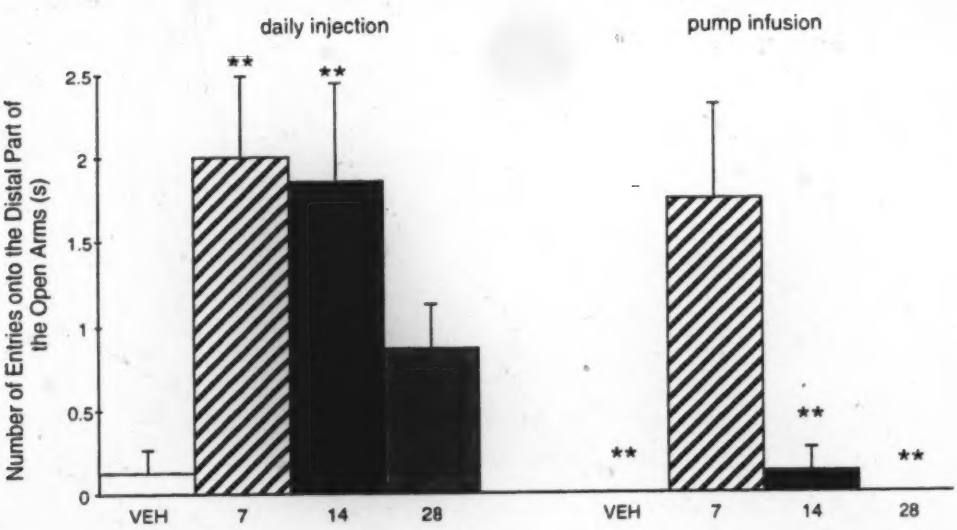


Figure 5.13:

The number of entries onto the distal portion of the open arms. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean ± SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. ** represents $P<0.01$, relative to the vehicle controls in the injection group and for the pump infusion group the differences are relative to the 7 day treatment group.

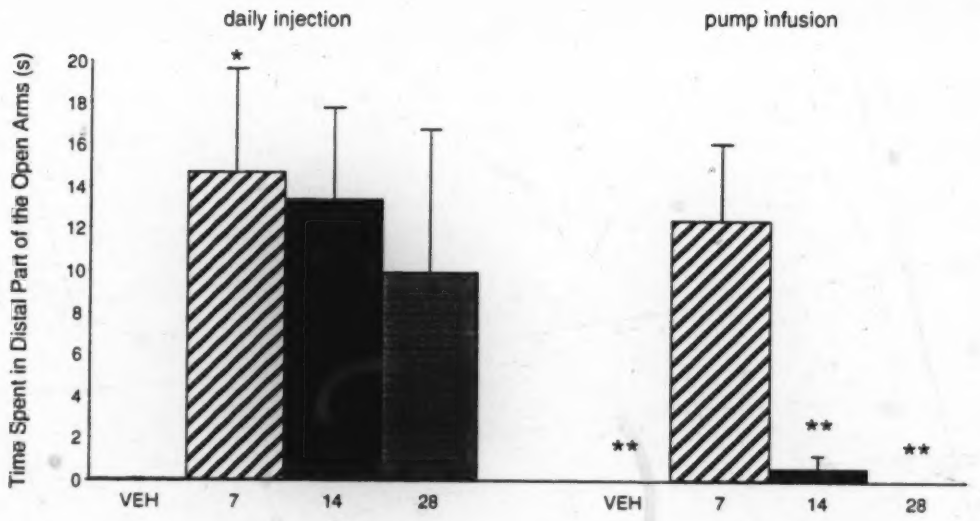


Figure 5.14:

The time the animal spent in the distal portion of the open arms. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. * represent $P<0.05$ and ** represents $P<0.01$. There was a significant difference between the vehicle controls and the 7 day treatment group in the injection dosing paradigm. For the pump infusion group the differences were relative to the 7 day diazepam infusion group.

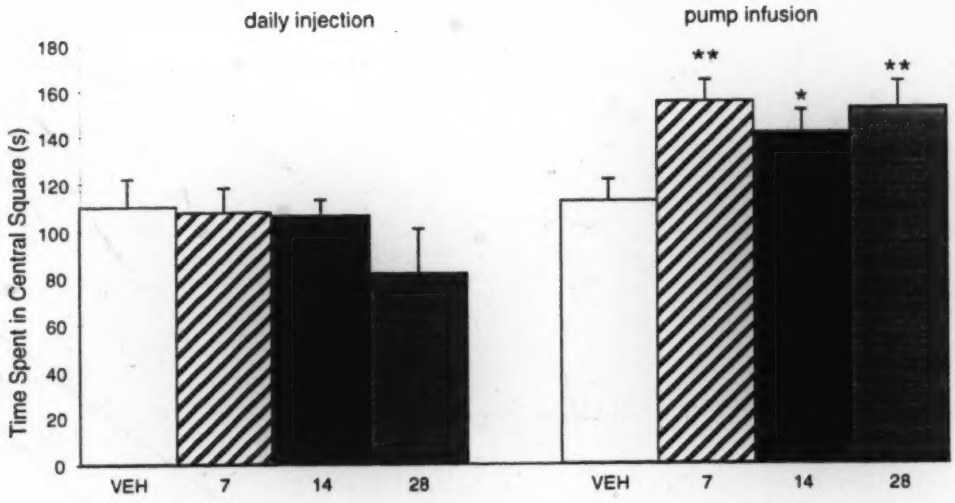


Figure 5.15:

The time the animal spent in the central square (s) of the plus maze. Acutely treated animals were not included in this analysis due to sedation affecting anxiolytic measurements. Plotted values represent the mean±SEM; VEH is vehicle control group and AC is the acute control group. In the injection treatment group the mean values were calculated from 7 or 8 test animals (n=8 for vehicle group). For the pump infusion group mean values were determined from 6-8 test animals (n=6 for vehicle group). Statistical analysis was performed using a Kruskal-Wallis, followed by Mann Whitney-U tests. * represent $P<0.05$ and ** represents $P<0.01$, relative to the vehicle controls.

CHAPTER 6

Differential effects of chronic full and partial agonist benzodiazepine treatment: alterations in $GABA_A$ receptor mRNA levels and binding characteristics in cortex, cerebellum, and hippocampus.

Introduction

Benzodiazepines can be classified according to their intrinsic activity or efficacy at the $GABA_A$ receptor (see Chapter 1). The full agonist diazepam has a high intrinsic activity and is highly efficacious, and therefore a range of receptor occupancy results in a spectrum of pharmacological effects. Partial agonists, however, have low intrinsic activities, or lower efficacy and induce a smaller fractional response in target cells compared to the full agonists at the same fractional receptor occupancy (Haefely et al., 1990; Facklam et al., 1992a,b; Mandema et al., 1992; Puia et al., 1992; Doble et al., 1993; Finn and Gee, 1993; Giusti et al., 1993; Maksay 1993). This spectrum of full agonist benzodiazepine effects include anxiolysis, sedation, myorelaxation, and seizure protection. However, the partial agonist pharmacological profile primarily includes anxiolysis and anticonvulsant action, even at receptor saturation (Chapter 1).

As mentioned previously, full agonist benzodiazepines are widely prescribed, yet with daily use tolerance develops to aspects of their pharmacological profile, notably the sedative and anticonvulsant effects. Nevertheless, partial agonist benzodiazepine chronic treatment does not appear to exhibit tolerance development.

The full and partial agonist benzodiazepines and benzodiazepine-like compounds (the cyclopyrrolones) interact with a specific recognition site on $GABA_A$ receptors in the mammalian CNS (Chapter 1). This receptor is an oligomer composed of multiple subunits, and previous studies have revealed that the specific pharmacological characteristics of a particular $GABA_A$ receptor subtype are defined by the subunit combination (Chapter 1; for review see Sieghart, 1995).

Chronic treatment with diazepam, or other full agonists, results in the development of tolerance, and has been shown to alter $GABA_A$ receptor affinity, function and allosteric modulation. Diazepam (Chapter 3; Chapter 4) has previously been shown to modify $GABA_A$ receptor mRNA levels to some degree. Chronic treatment with partial agonists

have also been examined to some extent. There is no indication of cross tolerance between chronic diazepam (Martin et al., 1995) and bretazenil treatment. Hernandez and colleges (1989) demonstrated a slight decrease in the sensitivity to GABA after chronic bretazenil treatment, which was dissociated from anticonvulsant tolerance, yet there were no changes in benzodiazepine binding characteristics. In Sf9 cells expressing specific $GABA_A$ receptor subtype $\alpha1\beta2\gamma2$, bretazenil treatment (60 hour exposure) caused a slight decrease in flunitrazepam binding, and when these cells expressed $\alpha5\beta2\gamma2$, bretazenil treatment resulted in a decrease in GABA potentiation that was similar to that seen following diazepam treatment (Primus et al., 1996). In mice chronically exposed to bretazenil, there was no development of anticonvulsant tolerance (Haigh and Feely, 1988), no tolerance to anxiolytic effects, no changes in TBPS binding or stimulated chloride uptake, but there was an decrease in specific binding of [^{3}H]-Ro 15-1788 in the cortex after 7 days treatment, which returned to normal after 14 days bretazenil (Miller et al., 1990). Recently, chronic treatment of rats with the partial agonist imidazenil demonstrated no changes in specific mRNA levels in certain areas of the cortex, cerebellum, and hippocampus, concurrent with no tolerance development to the bicuculline seizure threshold (Impagnatiello et al., 1996).

This chapter demonstrates, using doses that result in high receptor occupancy, that after 14 days chronic daily s.c. injection with diazepam (full agonist), bretazenil or RP 60503 (partial agonists), there are differential alterations in the expression of $GABA_A$ receptor genes and in the subsequent binding characteristics of $GABA_A$ receptors. Chronic exposure to these drugs demonstrates unique fingerprints of $GABA_A$ receptor gene expression and benzodiazepine binding capacity that differ amongst brain regions. Moreover, there are specific differences between diazepam, which causes tolerance, and the partial agonists, bretazenil and RP 60503, which do not.

Materials and Methods

Drug Treatment

For animal habituation see Chapter 3. For chronic drug administration rats were injected subcutaneously (s.c.) daily with either 15 mg/kg diazepam, 0.2 mg/kg bretazenil, or 7.5 mg/kg RP 60503 (dissolved in a maximum of 1 ml 50% DMSO/50% PG (v/v) vehicle) or vehicle alone. Rats were treated for 14 days, then sacrificed, and brains dissected and stored until use as described in Chapter 3.

Diazepam and bretazenil were generous gifts from Hoffmann-La Roche, Basel, Switzerland and RP 60503 was a generous gift from Rhône Poulenc Rourer, Paris, France.

Quantification of drug levels by reversed-phase HPLC

Diazepam and Bretazenil

Diazepam levels were quantified as previously described in Chapter 3. This method was also used for quantification of bretazenil, with the exception that ultraviolet absorption of the bretazenil eluant was monitored at 235 nm. Flunitrazepam (100 ng/50 µl injection) was used as the internal standard for both assays.

RP 60503

RP 60503 levels were quantified by Gail Rauw in the laboratory of Dr. Glen Baker, Department of Psychiatry, University of Alberta. Cortical tissue was homogenized in 5 volumes ice cold methanol, centrifuged at 12000 x g for 15 minutes, dried under nitrogen gas and reconstituted in 200 µl of mobile phase. A 20 µl aliquot was injected onto a 25 cm x 0.46 cm Hypersil 5 CN column (Phenomenx, California, U.S.A.) and eluted using an isocratic gradient of 25% (v/v) acetonitrile with 0.01 M ammonium acetate buffer, pH 5.4, over 30 minutes with a flow rate of 1.5 ml/min. Fluorescence detection of the eluant was at 470 nm; trazadone (5 ng/20 µl injection) was used as the internal standard.

RNA Isolation and Solution Hybridization

These methods were performed as previously described in Chapter 3.

Densitometry and Statistical Analysis of mRNA Steady-State Changes

Gels were dried, fixed and quantified according to the methods of Chapter 3. Mean relative mRNA levels were normalized to vehicle treated controls and compared between treatment groups within a subunit class. Statistical tests applied included a one-way ANOVA within each subunit class (comparing treatment groups) followed by the Student Newman-Keuls. The statistical test was performed within each separate subunit class because differences in the abundance levels for subunit isoforms affect the variances of measurements. Generally animal groups ranged in numbers from 4-6 and determinations were repeated 3-4 times. There were no inter-assay differences between separate experiments, and this allowed the data sets to be combined (see Appendix 1).

Binding Analysis

Binding assays were performed as described in Chapter 4. A one-way ANOVA was similarly applied, followed by a Student Newman-Keuls comparing treatment regimes (including vehicle treatment) within each group of binding assay experiments. The n values ranged within each assay from 3-6 animals. Assays were performed in either duplicate or triplicate and protein concentrations were normalized to 0.75 mg/ml as previously described (Chapter 4).

Results

Drug Levels

Drug levels were measured 24 hours after the last injection. Diazepam levels are as reported in Chapter 3. Briefly, after 14 days daily s.c. injection with 15 mg/kg diazepam, cortical levels are 106.3±9.3 (mean ± SEM; n=9) ng/g cortex. Further, as previously described (Chapter 4), a single daily s.c. injection of 15 mg/kg diazepam results in an initial increase in drug level, with maximal concentration occurring 2 hours post injection (Figure 6.1a). The bretazenil cortical level after 14 days chronic daily s.c. injection with 0.2 mg/kg/day was 40.2±7.0 ng/g (n=8). Bretazenil levels did not reach a maximum until 4 hours after a single injection (195.9±6.9 ng/g cortex, n=2) and rapidly declined to below detectable levels 12 hours post-injection (Figure 6.1a). RP 60503 levels reached maximal at 4 hours post single injection (52.2±5.1 ng/g cortex, n=2), but they were below the level of detection 8 hours after administration (Figure 6.1a). After 14 days s.c. injection with 7.5 mg/kg RP 60503 drug levels could not be consistently detected 24 hours after the last injection.

Changes in Steady-State mRNA Levels

Cortex

$GABA_A$ receptor mRNA steady-state levels after diazepam treatment are as reported in Chapter 3. There was a significant increase in α3-, α5- and β3-subunit mRNA levels after diazepam treatment (108±3.7%, 110.6±8.9%, and 122.5±6.9%; mean ± SEM) compared to the mean steady-state mRNA levels after treatment with bretazenil (95.5±3.1%, 84.9±3.2%, and 102.4±4.7%) or RP 60503 (91.5±4.4%, 86.6±5.2%, and 81.5±6.0%; Figure 6.2). Further, there was a significant decrease in β2- and γ3-subunit mRNA levels after 14 days of diazepam (92.3±3.0% and 96.5±8.9%) relative to the steady-state mRNA levels after chronic partial agonist bretazenil (107.5±3.1% and

118.5±10%) or RP 60503 (108.3±4.5% and 130.1±3.5%) treatment (the 20% decrease in γ3-subunit mRNA level between diazepam and bretazenil treatment failed to reach significance). In contrast, partial agonist treatment differentially altered α2- and β1-subunit $GABA_A$ receptor gene expression; RP 60503 treatment caused a significant decrease (84.2±4.8%) in α2-subunit mRNA level relative to vehicle, bretazenil (110.1±6.2%) and diazepam (99.3±3.9%) steady-state mRNA levels. The β1-subunit mRNA level was 76.9±6.2% after bretazenil treatment; this was significantly decreased compared to vehicle, RP 60503 (103.9±10.2%) and diazepam (106.2±8.3%) treatment.

Cerebellum

α1-, α2-, α4-, α5-, and γ1-subunit $GABA_A$ receptor mRNA levels were significantly different between daily diazepam and partial agonist treatment groups (Figure 6.3). The mean steady-state mRNA levels for the α1 subunit were 100.4±3.1% after diazepam treatment versus 88.3±4.7% and 88.2±3.8% after bretazenil and RP 60503 treatment, respectively. Further, both partial agonists significantly decreased in α1-subunit mRNA levels compared with vehicle. There was a significant increase in γ1-subunit mRNA levels after diazepam (106.5±7.2%) treatment versus bretazenil (80.4±9.4%) and RP 60503 (74.2±10.5%) treatment. This decrease in γ1-subunit steady-state mRNA level after RP 60503 treatment was also significant relative to vehicle. The mean steady-state mRNA levels after diazepam treatment were 73.8±9.3%, 61.5±9.2%, 69.1±11.7% for the α2-, α4-, and α5-subunits; these were significantly decreased relative to vehicle and partial agonist treatment. α2-, α4-, and α5-subunit mRNA levels were 109.4±7.4%, 92.0 5.9%, and 127.5±7.9% after bretazenil treatment and 102.8±7.7%, 108.0±8.7% and 108.9±9.7% after RP 60503 treatment, respectively. The β1- and γ3-subunit steady-state mRNA levels after diazepam were significantly decreased (86.1±8.5% and 81.9±5.6%) relative to bretazenil treatment (109.8±4.5% and 122.9±11.6%), and after RP 60503 treatment, this reduction did not reach significance. The only steady-state mRNA alteration in the cerebellum that differed between the partial agonist treatments was the β2-subunit, where

RP 60503 caused a significant increase (123.8±7.5%) relative to vehicle, bretazenil (96.6±4.6%) and diazepam (102.9±4.3%) treatment.

Hippocampus

There were significant differences between diazepam and partial agonist treatment in the hippocampal α5-, β2-, and β3- $GABA_A$ receptor mRNA subunit isoforms (Figure 6.4). The α5-subunit mRNA level was significantly decreased to 87.8±3.3% after diazepam treatment relative to bretazenil (105.2±2.1%) and RP 60503 treatment (95.6±2.0%); further, this RP 60503 treatment induced decrease was also significant relative to the bretazenil treatment. Partial agonist treatment significantly increased the β3-subunit steady-state mRNA level relative to both vehicle and diazepam treatment. β3-subunit mRNA levels were 130.6±7.9% and 124.5±6.4% after bretazenil and RP 60503 treatment, respectively, and after diazepam treatment the β3-subunit mRNA level was 83.8±7.6%. In contrast, there was an increase in β2-subunit expression after diazepam treatment (113.8±3.3%) versus vehicle, bretazenil (102.6±3.7%) and RP 60503 (102.1±3.1%) regimes. The level of α4-subunit mRNA was significantly decreased to 76.8±10.4% after diazepam treatment relative to the vehicle control treatment, whereas there were no significant difference after partial agonist exposure. Chronic treatment with RP 60503 showed a large significant increase in α2-, β1-, γ1-, and γ2-subunit gene expression relative to vehicle, bretazenil and diazepam dosing regimes. The steady-state mRNA levels for these subunits after RP 60503 treatment were 147.9±7.3%, 187.8± 8.8%, 127.7±4.4%, and 148.7±7.6%, respectively. Steady-state levels, after bretazenil treatment, were 97.4±5.8%, 118.9±10.1%, 103.0±6.1%, and 118.6±5.3%, which were similar to the steady-state mRNA levels after diazepam treatment (113.5±7.4%, 128.8±6.1%, 105.6±6.5% and 110.1±8.9%, respectively).

$GABA_A$ Receptor -Benzodiazepine Binding Analysis

The binding profile for cortical membrane preparations from animals which received diazepam treatment have been reported previously in Chapter 4.

Benzodiazepine Subtype Ratios

After chronic 14 day treatment with bretazenil, RP 60503, diazepam or vehicle, the ratio of BZ2/BZ1 sites was significantly increased in cortical membrane preparations from animals treated with the partial agonists (0.631± 0.02 and 0.662±0.033, respectively; mean ± SEM) compared to the diazepam (0.533± 0.012) and vehicle (0.527±0.011; Table 6.1) ratios. In the cerebellum, there were no significant alterations in BZ receptor subtype ratios (Table 6.2).

In hippocampal membrane preparations, partial agonist chronic treatment significantly decreased the BZ2/BZ1 ratio relative to both diazepam and vehicle ($P<0.05$) treated membrane preparations (Table 6.3). The ratio after treatment with bretazenil was 0.496±0.018 and after RP 60503 was 0.430±0.037, which were significantly lower than after diazepam (0.678±0.062) or vehicle (0.607± 0.02) treatment. This pattern was reversed in the BZ3/BZ2 ratio where there was a significant increase in this ratio following partial agonist treatment relative to diazepam and the vehicle control. The hippocampal membrane BZ3/BZ2 ratio from vehicle treated rats was 0.450± 0.023 and from diazepam treated animals this ratio was 0.460±0.041. However, after chronic partial agonist treatment, the BZ3/BZ2 ratio increased to 0.616±0.032 and 0.608±0.061 after exposure to bretazenil and RP 60503, respectively.

GABA Enhancement of [^{3}H]-Flunitrazepam Binding

In membrane preparations from both the cortex and cerebellum chronic drug treatment with either diazepam or the partial agonists resulted in a significant compromise of GABA enhanced [^{3}H]-flunitrazepam binding relative to membrane preparations from vehicle treated controls. In the cortex the increase in benzodiazepine binding as a result of

the addition of GABA was 151.2±1.6%; however, this shift was limited to 121.3±4.2% after diazepam, 119.6±2.7% after bretazenil and 119.1±2.3% after RP 60503 chronic treatment (Figure 6.5). In cerebellar vehicle membranes the GABA enhancement of binding was 141.7±3.4% which significantly declined to 118.5± 5.7%, 116.1±2.0%, and 122.1±4.8% after chronic treatment with diazepam, bretazenil, or RP 60503, respectively .

However, in hippocampal membrane preparations only chronic treatment with diazepam demonstrated a compromised GABA enhancement of [^{3}H]-flunitrazepam binding relative to vehicle membrane preparations. The increase in binding in vehicle control membranes was limited to 126.3±2.3%, which dropped to 109.2± 2.5% after diazepam chronic treatment. In membranes from bretazenil and RP 60503 chronically treated rats the enhancement in binding was 121.6±6.5% and 116.7±2.9%, respectively .

Total [^{3}H]-Ro 154513 Binding and Diazepam Insensitive Binding

In cortical membrane preparations there were no significant differences in total specific [^{3}H]-Ro 15-4513 binding between treatment regimes; however, there was a trend for an increase in binding after diazepam treatment. The amount of [^{3}H]-Ro 15-4513 binding was 661±15 fmol/mg after vehicle treatment which was slightly decreased after partial agonist treatment to 621± 38 fmol/mg with bretazenil and 636±49 fmol/mg with RP 60503 dosing. The binding after chronic diazepam treatment was elevated to 821 ± 82 fmol/mg (Figure 6.6a).

There was a significant increase in the relative proportion of diazepam insensitive (DIS) binding between membranes from chronic diazepam treated animals and membranes from chronic partial agonist treated rats. After vehicle treatment, the proportion of DIS binding was 1.9±0.87% of [^{3}H]-Ro 15-4513 binding, which decreased following bretazenil treatment to 1.17±0.25% and further to 0.89±0.36% after RP 60503 treatment. However, there was an increase in this DIS binding proportion to 2.62±0.31% of [^{3}H]-Ro 15-4513 binding after chronic diazepam exposure (Figure 6.6b).

In the cerebellum, there were no significant alterations in total specific [^{3}H]-Ro 15-4513 binding or the proportion of DIS binding between treatment regimes (Figure 6.7a/b). The amount of specific [^{3}H]-Ro 15 4513 binding in vehicle control animals was 339±15 fmol/mg and the relative amount that was DIS was 33.9±2.77%. After diazepam treatment, there was 294±21 fmol/mg binding and the DIS binding was 28.18±2.23%. Bretazenil partial agonist exposure resulted in a 34.34±2.88% DIS component with 276±6 fmol/mg radioligand binding and this was similar to the 33.82±1.76% DIS binding proportion of the 342±29 fmol/mg specific [^{3}H]-Ro 15-4513 binding in cerebellar membranes following RP 60503 treatment.

Similarly in the hippocampus there were no significant changes in total binding or DIS binding following any of the treatment regimes. The amount of specific [^{3}H]-Ro 15-4513 binding in the vehicle controls was 263±12 fmol/mg of which the relative amount of DIS binding was 2.93±0.38%. After chronic treatment with the benzodiazepine diazepam the amount of binding was 245±17 fmol/mg and after either exposure to bretazenil or RP 60503 the binding of specific [^{3}H]-Ro 15-4513 was 251±1 fmol/mg and 274±17 fmol/mg respectively. The relative proportion of DIS binding compared to total binding was 1.68± 0.34% after diazepam treatment, and 1.91±0.45% and 2.26±0.16% after bretazenil and RP 60503 exposure.

Discussion

The drug treatment regimes used in this study were predicted to result in similar high receptor occupancy levels (Doble et al., 1993; Martin et al., 1993). These paradigms were chosen to ensure that alterations which occurred consequently in the $GABA_A$ receptor were the result of differences in intrinsic efficacy and not differences in receptor reserve. The diazepam levels measured following treatment have been commented on previously in

Chapter 4 in more detail. Briefly, the diazepam level detected in the brain 2 hours post-injection was approximately 400ng/g (3% of the total daily dose administered, based per gram tissue in the brain with 15µg/g total dose) and after 24 hours was 0.2% of this daily amount could still be quantified. This prolonged retention of diazepam may result from the saturation of the degradative metabolic pathways (St.Pierre and Pand, 1995), or may be a result of the method of treatment (s.c. injection) which is not affected as strongly by first pass metabolism.

The peak concentration of bretazenil following a single injection occurred 4 hours after a single administration of 0.2 mg/kg, and rapidly dropped below the level of detection at 12 hours (Figure 6.1a). The peak concentration in the brain was 106 ng/g cortex and at 12 hours was 40ng/g cortex, just above the level of detection. The bretazenil dose resulted in high levels of $GABA_A$ receptor occupation, yet this low dose would not cause metabolic paths to be saturated. The proportion of drug measured in the brain was higher after bretazenil than diazepam treatment, but the bretazenil levels were not maintained beyond 12 hours. The pharmacokinetics of individual benzodiazepines have been reported to vary (Mandema et al., 1991); consequently, any differences between the pharmacokinetics of diazepam and bretazenil levels would not be surprising. The pharmacokinetics of bretazenil in the rat have not been extensively investigated; the mean residence time has been reported to be 48 minutes (Mandema et al., 1992) which is slightly longer than that of diazepam (41 minutes). In man, 6 metabolites have been identified, but the metabolism of bretazenil has not been extensively examined in the rat (Mandema et al., 1992).

The level of bretazenil in the brain after 14 days of chronic treatment was 40 ng/g. The amount of bretazenil detected in the brain was less than half of the diazepam amount measured (which was only 1% of the diazepam dose administered). A report suggests that this discrepancy may be the result of a high capacity metabolic pathway utilized after the primary saturation of the metabolic pathways by the high dose diazepam (St. Pierre and Pand, 1995). Secondly, although both compounds were given by the same route of

administration, the absorption and distribution of these compounds may differ. Moreover, the level of bretazenil in the cortex following daily s.c. injections for 14 days was similar to bretazenil levels detected in the brain after silastic pellet infusion for 21 days (Hernandez et al., 1989). Unfortunately, the daily dose administered by continuous infusion was not reported. The brain levels detected in this study confer no information about the drug concentrations in the rest of the body.

RP 60503 cortical drug levels were determined 1-8 hours post single administration, and although the maximal concentration was 52 ng/g cortex (0.6% of the administered dose), reached at 4 hours, there was no definitive maximal level. The recovery of this extraction assay was 75%, however, in all treated samples assayed there was an unknown peak detected. This peak may possibly be a metabolite, as it was not seen in the standards following methanol extraction, however a similar peak was found when the standards were basified (pH 11.5) in the first extraction attempt. Analysis of time course samples basified demonstrated only the unknown peak, with no RP 60503 peak being detected. The cortical samples were stored for a variable time after animal sacrifice, before quantification of drug levels, therefore there was a concern about the breakdown of the compound. Hence, the 1 hour post injection regime was repeated and fresh cortex was extracted in methanol and analyzed. This fresh sample revealed an RP 60503 level of 100.7±7.5 ng/g cortex (n=3; Figure 6.1b), (1.3% of the administered dose) which was twice as much as the previous 1 hour sample that had been stored for up to 18 months; this non-stored sample also demonstrated the additional unknown peak. This information, in combination with erratic peaks during gas chromatography attempts, indicate that there was a possible artifact during quantification, or an RP 60503 metabolite may have formed in the rat. Nonetheless this compound appeared to be unstable after long term storage.

After chronic daily injections of these compounds, there were brain region and drug specific changes in both the expression of $GABA_A$ receptor genes and in the corresponding benzodiazepine binding capacities of the subsequent receptors. Brain region specific

$GABA_A$ receptor mRNA changes resulting from diazepam exposure have been previously described in this thesis (Chapter 3) and in the literature (Primus and Gallager, 1992; Impagnatiello et al., 1996; Holt et al., 1996). The present study demonstrated that the development of brain region specific changes were also extended to other benzodiazepines and benzodiazepine-like compounds. For instance, in the cortex, chronic diazepam treatment resulted in an increase in the level of α5-subunit mRNA, whereas in the cerebellum and hippocampus, there was a significant decrease in α5-subunit isoform gene expression. This pattern was also observed after chronic RP 60503 treatment: there was a significant decrease in α2-subunit mRNA level in the cortex whereas this subunit isoform was significantly increased in the hippocampus. This brain region specificity was also observed in the ratios of the BZ receptor subtypes: after chronic partial agonist treatment there was a significant increase in the BZ2/BZ1 ratio in cortical membrane preparations relative to vehicle preparations, and this was opposite to that observed in the hippocampal membrane preparations where the BZ2/BZ1 ratio was significantly decreased.

This chapter has also demonstrated that alterations in $GABA_A$ receptor gene expression and corresponding benzodiazepine binding recognition properties were specific to drug treatments which vary in their intrinsic efficacy. Patterns of change after chronic treatment with the full agonist diazepam differed relative to chronic treatment with the partial agonists bretazenil and RP 60503. In the cortex, this differential pattern was observed for the α3-, α5-, β2-, β3-, and γ3-subunit mRNA levels; chronic full agonist treatment caused an increase in mRNAs in α3-, α5-, and β3-subunits and a corresponding decrease in these isoforms resulted after partial agonist treatment. The decrease in β2- and γ3-subunit expression after diazepam treatment was opposite to that detected after bretazenil and RP 60503 treatment. This observation can be extended to the cerebellum with the α1-, α2-, α4-, α5-, β1-, and γ1-subunit isoform mRNA levels and to the hippocampus with β2- and β3- subunit isoforms.

Benzodiazepine binding properties demonstrated changes that were opposite between full agonist and partial agonist treatment. For example, in cortical and hippocampal membrane preparations, there were significant differences between partial agonist treatment and diazepam treatment in the BZ2/BZ1 ratio. In the cortex, chronic partial agonist treatment regimes resulted in an increase in this ratio relative to vehicle and to diazepam treatment whereas in the hippocampus bretazenil and RP 60503 chronic administration resulted in a significant decrease in the BZ2/BZ1 ratio. These oppositional effects may reflect the distinct circuitry of the various brain regions, and how diverse modulation may differentially alter particular GABAergic "wiring".

Drug specific changes also occurred between the two partial agonist treatments. There were significant differences between bretazenil and RP 60503 treatment regimes in the α2- and β1-subunit mRNA levels in the cortex; α4-, β2-, and γ3-subunits in the cerebellum and in the α2-, α5-, β1-, γ1-, and γ2-subunit mRNA levels in the hippocampus. A differential decrease in the BZ3/BZ2 ratio between the partial agonists was also found, but did not reach significance. In some instances, partial agonist treatment differed in $GABA_A$ receptor gene expression. Generally, when these compounds varied, the bretazenil treatment altered $GABA_A$ receptor gene expression similar to the diazepam treatment regime (i.e. β1-, γ1- and γ2-subunit mRNA levels in the hippocampus); these differences may be a result of the inconsistency between the intrinsic activities of the two partial agonists. Bretazenil is considered to have intermediate intrinsic activity (Martin et al., 1993) between the full agonist diazepam and RP 60503, the latter having a very low intrinsic activity (Doble et al., 1993). Further, the differences may result from variations in intrinsic efficacy at specific benzodiazepine receptor subtypes (Facklam et al., 1992a,b; Puia et al, 1992; Finn and Gee 1993; Maksay 1993: Mehta and Shank, 1995; Chen et al, 1996), in that bretazenil has been shown to have increased efficacy at α4-subunit containing $GABA_A$ receptors, compared to diazepam. The RP 60503 efficacy at the

various $GABA_A$ receptor subtypes is currently unknown (Knoflach et al., 1996; Wafford et al., 1996; Whittemore et al., 1996).

It has previously been reported that after bretazenil treatment, there were no changes in GABA's efficacy or affinity, but in contrast to chronic diazepam treatment, there was an increase in the GABA enhancement of [^{3}H]-flunitrazepam binding and a decrease in the specific binding of [^{3}H]-flunitrazepam (Hernandez et al, 1989). However, the author explained that these changes may have been a consequence of residual bretazenil remaining in the membrane preparation. This paper further showed that after chronic bretazenil there were subpopulations of neurons which were subsensitive to GABA in the dorsal raphe nucleus and which had an increased affinity for GABA, as measured by [^{3}H]-bicuculline binding. This subpopulation showed alterations similar to chronic full agonist treatment, and this subset corresponded to the ratio of animals which had a decreased bicuculline seizure threshold (after chronic bretazenil treatment). These differences may reflect the breadth of bretazenil treatment effects on heterogeneous receptor subtype populations (Hernandez et al., 1989).

Allosteric coupling between the GABA and benzodiazepine site can be measured by the enhancement of radiolabeled agonist benzodiazepine binding in the presence of GABA, termed GABA shift. In cortical and cerebellar membrane preparations, treatment regimes resulted in a decrease in GABA enhancement of [^{3}H]-flunitrazepam binding, in contrast to the hippocampus, where only diazepam treatment demonstrated a significantly compromised GABA shift. This lack of enhancement may indicate uncoupling between the binding sites, and may give an indication that benzodiazepine modulation would fail to increase GABA binding and henceforth, fail to increase neuronal inhibition. If this were the case, the chronic drug regimes examined in this study would be expected to have a decreased efficacy in the cortex and cerebellum. This study did not examine efficacy after chronic treatment; however extrapolations from the literature indicate this may be the case (Primus et al., 1996). Although GABA enhancement by RP 60503 in naive membrane

preparations demonstrated weaker enhancement than diazepam (Doble et al., 1993) and bretazenil was not as effective as diazepam at facilitating GABA-stimulated chloride influx or TBPS binding (Haefely et al., 1990; Facklam et al., 1992a; Finn and Gee 1993; Li et al. 1993; Maksay 1993), after extended treatment with bretazenil in the Sf9 expression system, flunitrazepam enhancement was similarly reduced (Primus et al., 1996). Therefore the allosteric uncoupling between the benzodiazepine and GABA-site, may not distinctly related to the intrinsic efficacy of ligands, and the development of tolerance, but may be due to the level of receptor occupation. In this study, measures were taken to ensure equivalent receptor occupancy, however receptor saturation may not be a physiological event with the partial agonists, and this paradigm used may have forced similarities with diazepam. In particular bretazenil and RP 60503 have a greater affinity than diazepam at the $GABA_A$ receptor, and therefore, to elicit their pharmacological effects, high levels of receptor occupancy would not generally be required. Furthermore, the exact $GABA_A$ receptor subtypes responsible for the compromised GABA shift were not revealed, and therefore the exact efficacies of these compounds on those receptor subtypes are unknown. Moreover, although the partial agonists reduced GABA enhancement upon chronic treatment, this effect may not be similar to diazepam's compromised shift, and may be dissociated from the induction of diazepam tolerance.

The specific binding of [^{3}H]-Ro 15-4513 and the relative proportion of diazepam sensitive and diazepam insensitive (DIS) binding in specific brain regions measure alterations in protein expression that occurred with chronic drug administration (see Chapter 4 for detailed explanation). In the cortex, the amount of specific [^{3}H]-Ro 15-4513 binding and the proportion of DIS binding corresponded to the pattern of α4-subunit expression after treatment with diazepam, bretazenil and RP 60503. Although the steady state mRNA levels and [^{3}H]-Ro 15-4513 binding alterations after these treatment regimes did not reach significance, there was a drug induced pattern. Furthermore, the cortical DIS binding increased significantly after chronic diazepam exposure relative to the partial

agonist treatment regime (Figure 6.6b). This increase in the DIS component after chronic diazepam treatment may represent an increase in the number of cortical $GABA_A$ receptors which are no longer sensitive to diazepam modulation.

In the cerebellum and hippocampus there were significant changes in α4-subunit mRNA levels; however, there were no significant alterations in the [^{3}H]-Ro 15-4513 binding or in relative DIS binding. However, as before, the patterns of change correspond; the magnitude of $GABA_A$ receptor protein expression change, measured by binding of radioligand, was not to the same extent as the $GABA_A$ receptor steady-state mRNA levels. In the cerebellum, the lack of alterations in α6-subunit gene expression may counteract the α4-subunit mRNA changes detected, and therefore no significant differences in binding can be measured. Further, it may be that the time courses for changes in protein expression vary and more time was needed for the alterations in $GABA_A$ receptor protein expression to be adjusted to the same degree as gene expression. Furthermore, it is possible that the observed alterations in the $GABA_A$ receptor gene expression were not dramatic enough to cause functional changes in the receptors, or alter benzodiazepine binding. Further, although binding has been utilized as a measure of $GABA_A$ receptor protein expression, it may not be sensitive enough and the binding assays may have missed the alterations. It is important to note, nonetheless, that the patterns of α4-subunit gene expression, [^{3}H]-Ro 15-4513 and DIS binding within each brain region correspond. This indicates, to a certain degree, that a correlation between gene expression and protein levels measured via benzodiazepine binding can be detected.

The percentage of benzodiazepine subtypes found via our zolpidem binding analysis correspond to percentages reported in the literature (Sieghart, 1995; Mehta and Shank, 1995). However, because a decrease or increase in total binding can alter the percentages of the BZ subtypes, the ratios of subtypes were examined to establish if there were any changes in the expression or pattern of BZ receptor subtype binding. Zolpidem, at various concentrations, was utilized to displace [^{3}H]-flunitrazepam in the 3 brain regions.

According to the distinct affinities for zolpidem by the benzodiazepine receptor subtypes containing different α-subunit isoforms, the proportion of BZ subtypes may be determined (see Chapter 4 and Figure 4.1).

In cortical membrane preparations, there was a significant increase in the BZ2/BZ1 ratio after chronic partial agonist treatment relative to diazepam and vehicle control treatment regimes. This alteration in binding ratio may indicate an increase in the BZ2 subtype component or a decrease in the BZ1 receptor subtype binding. This change was opposite to that which occurs in hippocampal membrane preparations, where the ratios indicate a decrease in the BZ2 subtype component or an increase in the BZ1 and BZ3 subtype after partial agonist chronic treatment. It is interesting to note, nonetheless, a relative degree of coherence, because for both regions the two ratios implicate the BZ2 component as being primarily responsible for the subtype ratio alterations. These changes in binding capacity may correlate to changes in protein levels and it may be possible to directly extrapolate from the changes in α-subunit gene expression. However, the changes in $GABA_A$ receptor subunit mRNA levels (Figure 6.2 and 6.3) do not correlate well with the binding data with regard to α-subunit protein. Further, the BZ subtype ratios do not indicate how alterations in the expression of the β and γ subunits may change the specific BZ subtype ratios; β2- and γ1-subunit containing receptors have an increased zolpidem affinity relative to the other β/γ isoforms (Herb et al., 1992; Hadingham et al., 1993b; Wafford et al., 1993b; Ebert et al., 1994; Graham et al., 1996; and for review see Sieghart 1995). There was no correlation between the $GABA_A$ receptor α subunit mRNA levels and the BZ subtype binding ratios, suggesting that other subunits play a role in benzodiazepine binding characteristics, or perhaps that these mRNA data do not reflect protein alterations.

Physiologically, these changes in BZ subtype ratios may not be relevant; the degrees of change were small, and although significant, may not have altered the physiological state/function of the $GABA_A$ receptor. However, it may be that the small changes were important, but the subsequent effects were too subtle to be detected with the

acuity of techniques utilized in this study. It is intriguing that the primary changes in BZ subtype, occurred after chronic partial agonist treatment and not following full agonist treatment. This may indicate that something particular to $GABA_A$ receptor occupancy by the partial agonists caused changes in how these receptor subsequently "encounter" benzodiazepines. Therefore, partial agonists induce changes which were different to diazepam, and may not relate to the induction of tolerance.

The literature has illustrated, primarily with transient expression studies, that various benzodiazepines have altered efficacy and potency at $GABA_A$ receptors composed of different subunit isoforms (Puia et al., 1992; Hadingham et al., 1993a; Ebert et al., 1994; Sieghart for review 1995; Burgard et al., 1996; Graham et al., 1996; Hadingham et al., 1996; Knoflach et al., 1996; Wafford et al., 1996; Whittemore et al., 1996). It is important to mention that the changes reported in both the $GABA_A$ receptor mRNA levels and in the benzodiazepine binding capacity were the consequences of the partial agonists and the full agonist acting at a heterogeneous population of $GABA_A$ receptor subtypes. Therefore, each different drug may have different efficacies at different $GABA_A$ receptor subtypes. For example, in cells stably expressing $\alpha4\beta2\gamma2$, the bretazenil Ki value (Knoflach et al., 1996) was increased by greater than 40-fold relative to its Ki at $\alpha1\beta3\gamma2$ expressing cells (Hadingham et al, 1996). Further bretazenil potentiation of the GABA response by these α4-subunit expressing cells was greater than flunitrazepam and Ro 15-4513 potentiation (Wafford et al., 1996) and bretazenil potentiation of the GABA response at the $\alpha4\beta1\gamma2s$ receptors was approximately 2 times its modulation of $\alpha1\beta1\gamma2s$ expressing cells (Wafford et al., 1996). This increase in modulation by bretazenil at α4-subunit containing receptors has also been observed with the α6-subunit expressing receptors (Hadingham et al., 1996; Knoflach et al., 1996).

Therefore, in situations where full agonist classical benzodiazepine modulation vastly decreases, partial agonist efficacy may increase, acting more as a full agonist than as a partial agonist, further complicating the examination of $GABA_A$ receptor subunit gene

expression as being a result of either full agonist (tolerance causing) or partial agonist manipulation. Inspection of the alterations in $GABA_A$ receptor α-subunit mRNA levels, with the varying efficacies of the ligands in mind, it is interesting that the α4-subunit mRNA levels in all 3 regions were altered in opposite directions after diazepam treatment relative to partial agonist treatment, with bretazenil continually the intermediate value. It may be suggested that some of the changes in $GABA_A$ receptor gene expression and subsequently the changes in benzodiazepine binding properties may not only be the resultant effects of the efficacy of these compounds on the $GABA_A$ receptor subtype, but of the interactions these ligands have on the GABAergic circuitry.

In conclusion, this chapter demonstrates that alterations which develop as a result of these treatment regimes correspond to differences between the full agonist diazepam, the intermediate partial agonist, bretazenil, and the weak partial agonist RP 60503, where in all instances receptor occupancy was equivalent. To determine the underlying mechanisms of tolerance it does not appear to be the simple task of determining what the differential changes are between diazepam and partial agonist chronic treatment, for each drug demonstrates its own profile, and within each profile there are comparisons which are similar among the benzodiazepines and those which are different. Partitioning these differences in the $GABA_A$ receptor mRNA and protein level is a complex tedious task which requires more knowledge about the $GABA_A$ receptor subtype *in vivo*.

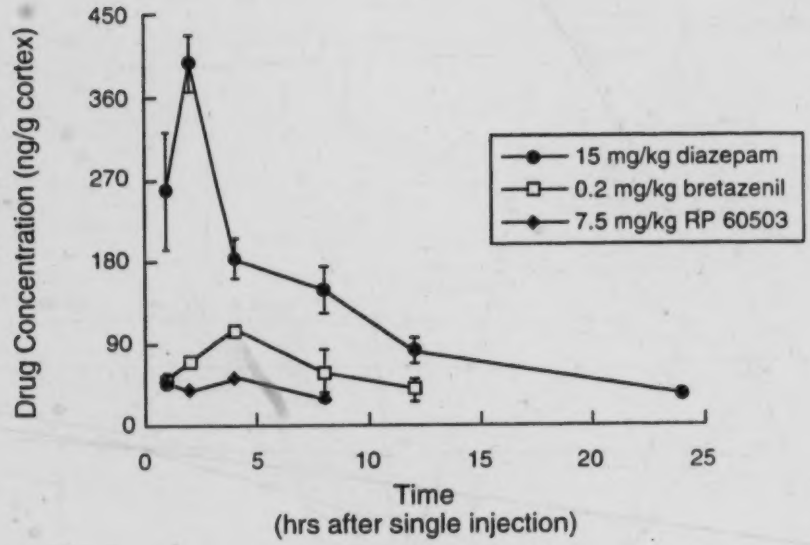
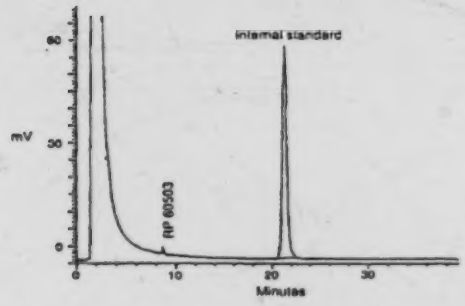


Figure 6.1:

A.) The kinetics of drug concentrations (ng/g cortex) in the rat measured by HPLC. Each point represents the mean drug concentration for 2-3 rats sacrificed at a specific time after s.c. injection with less than 1.0 ml of 50% DMSO/50% PG (v/v) containing either 15 mg/kg diazepam, 0.2 mg/kg bretazenil or 7.5 mg/kg RP 60503. Error bars represent the SEM and those error bars not seen are within the symbol. Bretazenil levels were below the level of detection after 12 hours and RP 60503 levels were below the level of detection after 8 hours.

I



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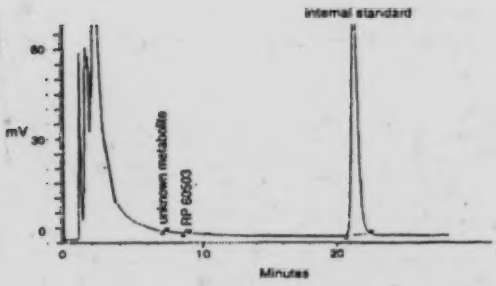


III

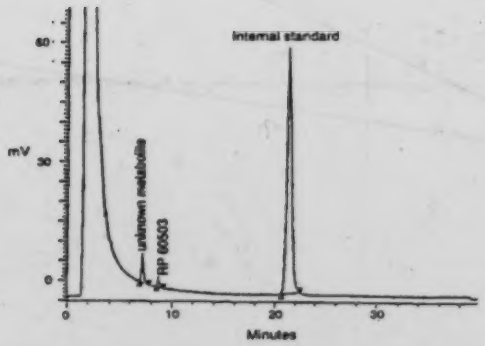
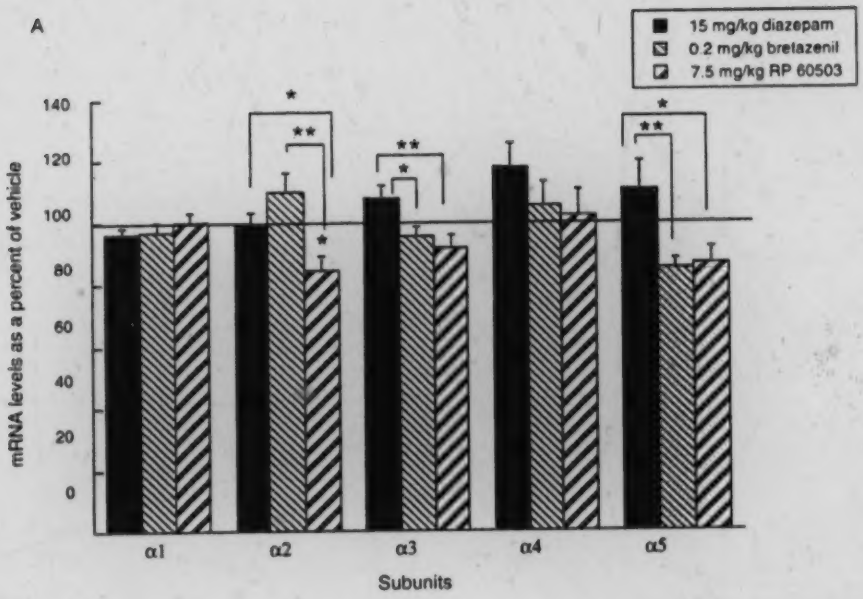


Figure 6.1:

B.) Sample RP 60503 HPLC peaks after methanol extraction from a 25 ng standard (I), from a 1 hour time point stored sample (II), and a fresh 1 hour time course sample that was not stored (III).

Figure 6.2:

Mean cortical $GABA_A$ receptor α-subunit (a) and β- and γ-subunit (b) steady-state mRNA levels as a percentage of vehicle treated controls after 14 days of daily treatment with 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatches), or 7.5 mg/kg RP 60503 (large hatches). Error bars represent SEM calculated from 2-4 independent S1 Nuclease assays performed on 4-6 animals per treatment group. mRNA measurements were normalized to a β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one way ANOVA followed by Student Newman-Keuls post ANOVA test to compare the treatment regimes.


A
15 mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA levels as a percent of vehicle
140
120
100
80
60
40
20
0
α1
α2
α3
α4
α5
Subunits

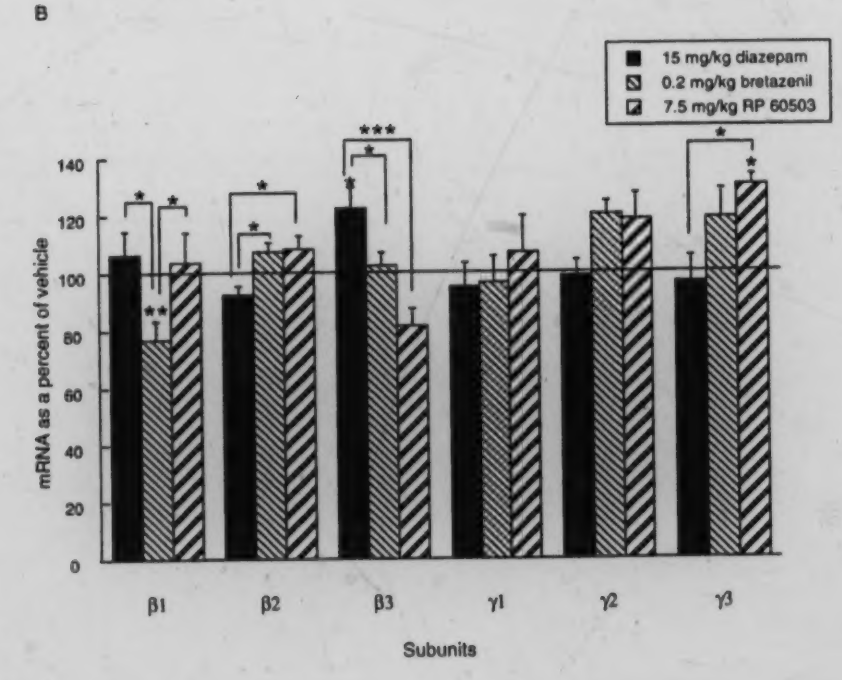
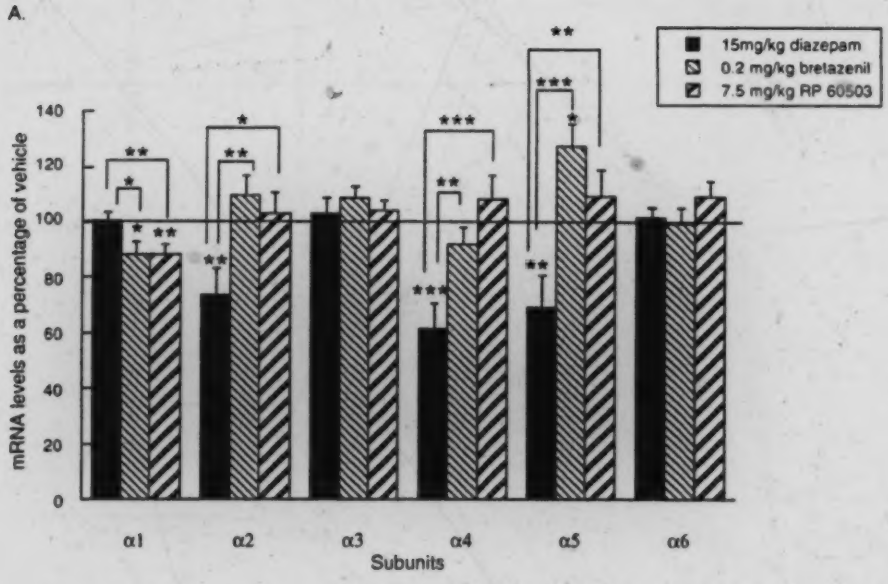

B
15 mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA as a percent of vehicle
140
120
100
80
60
40
20
0
β1
β2
β3
γ1
γ2
γ3
Subunits

Figure 6.3:

Mean cerebellar $GABA_A$ receptor α-subunit (a) and β- and γ-subunit (b) steady-state mRNA levels as a percentage of vehicle treated controls after 14 days of daily treatment with 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatches), or 7.5 mg/kg RP 60503 (large hatches). Error bars represent SEM calculated from 2-4 independent S1 Nuclease assays performed on 4-6 animals per treatment group. mRNA measurements were normalized to a β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one way ANOVA followed by Student Newman-Keuls post ANOVA test to compare the treatment regimes.


A.
15mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA levels as a percentage of vehicle
0
20
40
60
80
100
120
140
α1
α2
α3
α4
α5
α6
Subunits

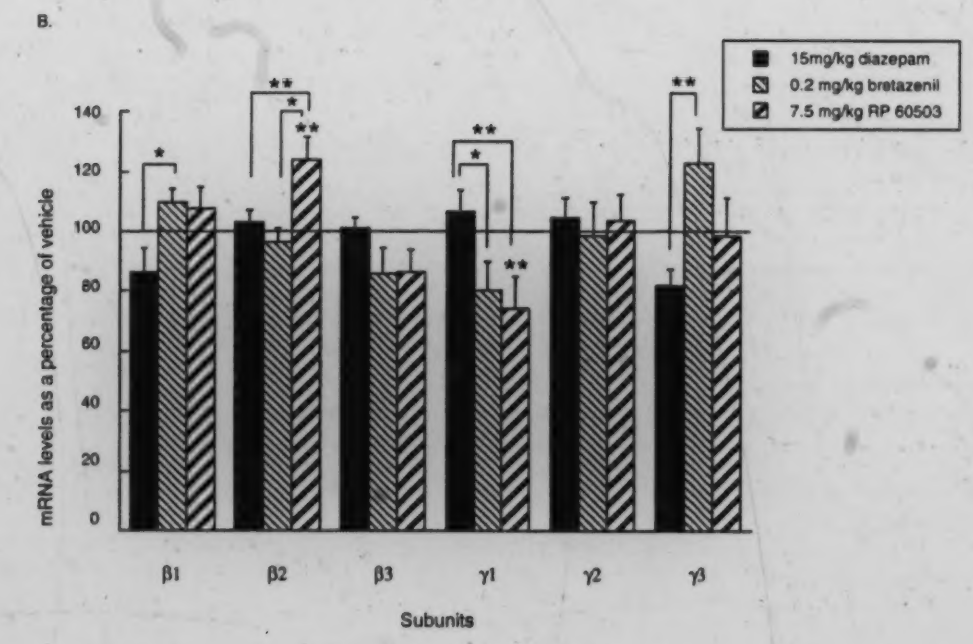
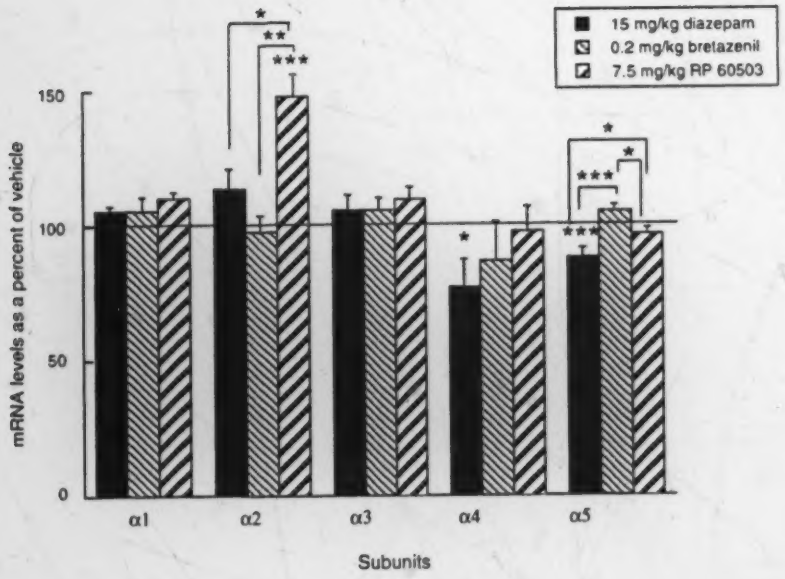

B.
15mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA levels as a percentage of vehicle
0
20
40
60
80
100
120
140
β1
β2
β3
γ1
γ2
γ3
Subunits

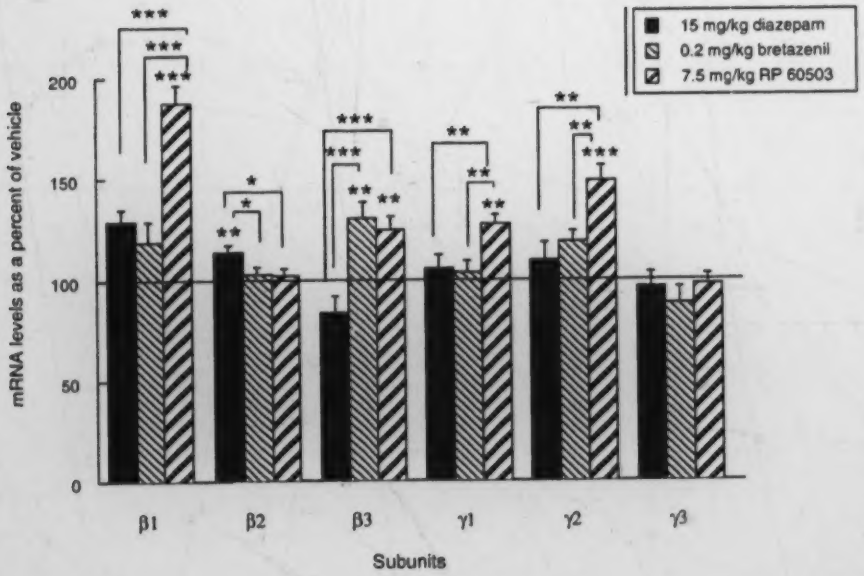
Figure 6.4:

Mean hippocampal $GABA_A$ receptor α-subunit (a) and β- and γ-subunit (b) steady-state mRNA levels as a percentage of vehicle treated controls after 14 days of daily treatment with 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatches), or 7.5 mg/kg RP 60503 (large hatches). Error bars represent SEM calculated from 2-4 independent S1 Nuclease assays performed on 4-6 animals per treatment group. mRNA measurements were normalized to a β-actin internal standard. * represents $P<0.05$, ** represents $P<0.01$ and *** represents $P<0.001$ using a one way ANOVA followed by Student Newman-Keuls post ANOVA test to compare the treatment regimes.

A.


15 mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA levels as a percent of vehicle
150
100
50
0
α1
α2
α3
α4
α5
Subunits

B.


15 mg/kg diazepam
0.2 mg/kg bretazenil
7.5 mg/kg RP 60503
mRNA levels as a percent of vehicle
200
150
100
50
0
β1
β2
β3
γ1
γ2
γ3
Subunits

Cortex	Benzodiazepine Subtypes			Benzodiazepine Subtype Ratios	
Treatment	BZ 1 (% of total)	BZ 2 (% of total)	BZ 3 (% of total)	BZ2/BZ1	BZ3/BZ2
vehicle (4)	60.1 ± 1.0	31.6 ± 0.4	8.3 ± 1.0	0.527 ±0.011	0.262 ± 0.033
diazepam (6) (15 mg/kg/day)	60.4 ± 0.4	32.2 ± 0.5	7.4 ± 0.3	0.533 ± 0.012	0.231 ± 0.011
bretazenil (6) (0.2 mg/kg/day)	56.7 ± 0.8	35.7 ± 0.7	7.6 ± 0.5	0.631 ± 0.02*#	0.214 ± 0.014
RP 60503 (6) (7.5 mg/kg/day)	55.9 ± 1.1	36.8 ± 1.1	7.3 ± 0.3	0.662±0.03**#	0.201 ± 0.012

Table 6.1:

The mean percentage of BZ subtypes from total binding and the relative ratios of these subtypes in cortical tissue from animals treated chronically with 15 mg/kg/day diazepam, 0.2 mg/kg/day bretazenil, 7.5 mg/kg/day RP 60503 or vehicle (50% DMSO/50% PG). Error represents SEM calculated from binding assays done in triplicate, with n=4-6. * represents $P<0.05$ for bretazenil versus vehicle BZ2/BZ1 ratio. ** represents $P<0.01$ for RP 60503 versus vehicle BZ2/BZ1 ratio. # represents $P<0.01$ for RP 60503 and bretazenil versus diazepam for BZ2/BZ1 ratio. Statistical analysis was performed using one way ANOVA followed by the Student Newman-Keuls post ANOVA test.

Cerebellum	Benzodiazepine Subtypes			Benzodiazepine Subtype Ratios	
Treatment	BZ 1 (% of total)	BZ 2 (% of total)	BZ 3 (% of total)	BZ2/BZ1	BZ3/BZ2
vehicle (6)	82.2 ± 1.3	16.3 ± 1.5	1.5 ± 0.6	0.200 ±0.022	0.108± 0.053
diazepam (6) (15 mg/kg/day)	83.1 ± 1.4	15.0 ± 1.5	1.9 ± 0.2	0.183 ± 0.021	0.133 ±0.02
bretazenil (6) (0.2 mg/kg/day)	83.5 ± 0.6	13.7 ± 0.6	2.8 ± 0.1	0.165 ± 0.009	0.204 ± 0.016
RP 60503 (6) (7.5 mg/kg/day)	82.3 ± 0.6	16.4 ± 0.5	1.3 ± 0.3	0.200 ± 0.007	0.080 ± 0.020

Table 6.2:

The mean percentage of BZ subtypes relative to total binding and the ratios of these subtypes in cerebellar tissue from animals treated chronically with 15 mg/kg/day diazepam, 0.2 mg/kg/day bretazenil, 7.5 mg/kg/day RP 60503 or vehicle (50% DMSO/50% PG). Error represents SEM calculated from binding assays done in triplicate, with n=4-6. Statistical analysis was performed using a one way ANOVA followed by the Student Newman-Keuls post ANOVA test.

Hippocampus	Benzodiazepine Subtypes			Benzodiazepine Subtype Ratios	
Treatment	BZ 1 (% of total)	BZ 2 (% of total)	BZ 3 (% of total)	BZ2/BZ1	BZ3/BZ2
vehicle (6)	53.3 ± 0.7	32.3 ± 0.7	14.4 ± 0.5	0.607 ±0.020	0.448 ± 0.023
diazepam (6) (15 mg/kg/day)	50.9 ± 2.2	33.7 ± 0.5	15.4 ± 1.3	0.678 ± 0.062	0.460 ± 0.040
bretazenil (6) (0.2 mg/kg/day)	55.6 ± 0.8	27.5 ± 0.7	16.9 ± 0.7	0.496 ± 0.017**	0.616 ± 0.032*
RP 60503 (5) (7.5 mg/kg/day)	60.3 ± 1.3	25.6 ± 1.7	14.1 ± 0.4	0.430±0.037***	0.608 ± 0.061*

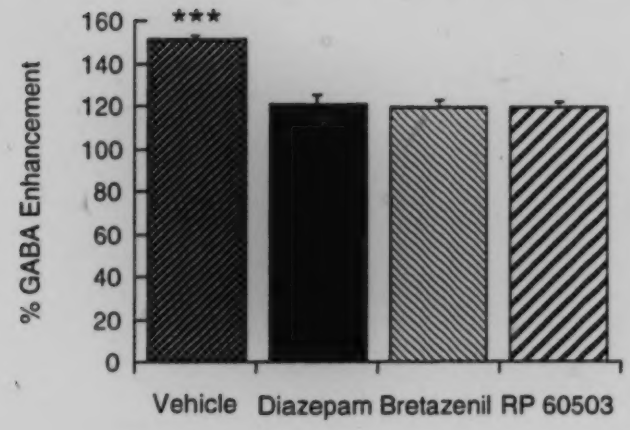
Table 6.3:

The mean percentage of BZ subtypes relative to total binding and the ratios of these subtypes in hippocampal tissue from animals treated chronically with 15 mg/kg/day diazepam, 0.2 mg/kg/day bretazenil, 7.5 mg/kg/day RP 60503 or vehicle (50% DMSO/50% PG). Error represents SEM calculated from binding assays done in triplicate, with n=4-6. *** represents P<0.001, ** represents P<0.01 and * represents P<0.05. RP 60503 BZ2/BZ1 ratio was decreased relative to diazepam, bretazenil BZ2/BZ1 ratio was decreased relative to diazepam, and both bretazenil and RP 60503 BZ2/BZ1 ratios were significantly different than vehicle controls (P<0.05). Bretazenil and RP 60503 BZ3/BZ2 ratios were significantly increased (P<0.05) relative to diazepam and vehicle. Statistical analysis was performed using a one way ANOVA followed by the Student Newman-Keuls post ANOVA test.

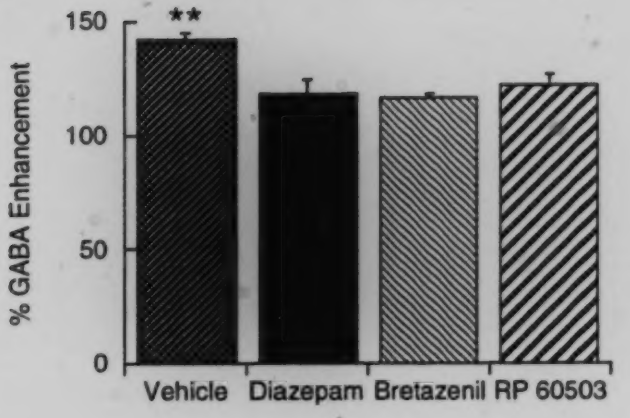
Figure 6.5:

The mean percentage of [^{3}H]-flunitrazepam binding enhancement after the addition of 100 μM GABA to cortical (a), cerebellar (b), and hippocampal (c) tissue preparations from animals treated with for 14 days with either 15 mg/kg/day diazepam (black), 0.2 mg/kg/day bretazenil (small hatched), 7.5 mg/kg/day RP 60503 (large hatched), or vehicle (black hatched). Error bars represent SEM calculated from binding analysis done in triplicate, with n=4-6. * represents $P<0.05$ after diazepam treatment, with a reduced GABA enhancement relative to the vehicle in the hippocampus. ** represents $P<0.01$ in a reduced GABA enhancement following chronic drug treatment compared to vehicle treated controls in cortical tissue. *** represents $P<0.001$ for significantly decreased GABA enhancement following chronic drug treatment, relative to vehicle controls in the cerebellum. Statistical analysis applied a one way ANOVA followed by a Student Newman-Keuls post ANOVA test comparing treatment regimes.

A.


***
160
140
120
100
80
60
40
20
0
% GABA Enhancement
Vehicle
Diazepam
Bretazenil
RP 60503

B.


**
150
100
50
0
% GABA Enhancement
Vehicle
Diazepam
Bretazenil
RP 60503

C.

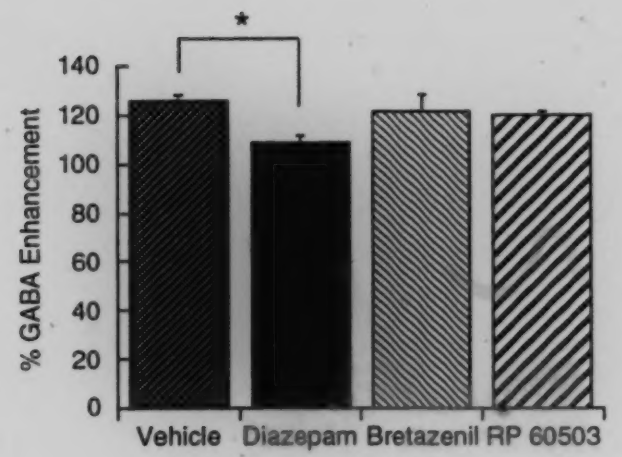

*
140
120
100
80
60
40
20
0
% GABA Enhancement
Vehicle
Diazepam
Bretazenil
RP 60503

Figure 6.6:

The mean total specific [^{3}H]-Ro 15-4513 binding (pmol/mg protein) (a) in cortical tissue from animals injected daily for 14 days with either 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatched), 7.5 mg/kg RP 60503 (large hatched), or vehicle (black hatched). Nonspecific binding was defined using cold Ro 15-4513. The mean proportion of [^{3}H]-Ro 15-4513 binding (DIS) after the addition of 10 μM diazepam (b). Error bars represent SEM calculated from binding assays done in triplicate, with n=4-6. * represents $P<0.05$ using one way ANOVA followed by a Student Newman-Keuls post ANOVA test. The DIS proportion after diazepam chronic treatment was significantly increased relative to partial agonist chronic treatment.

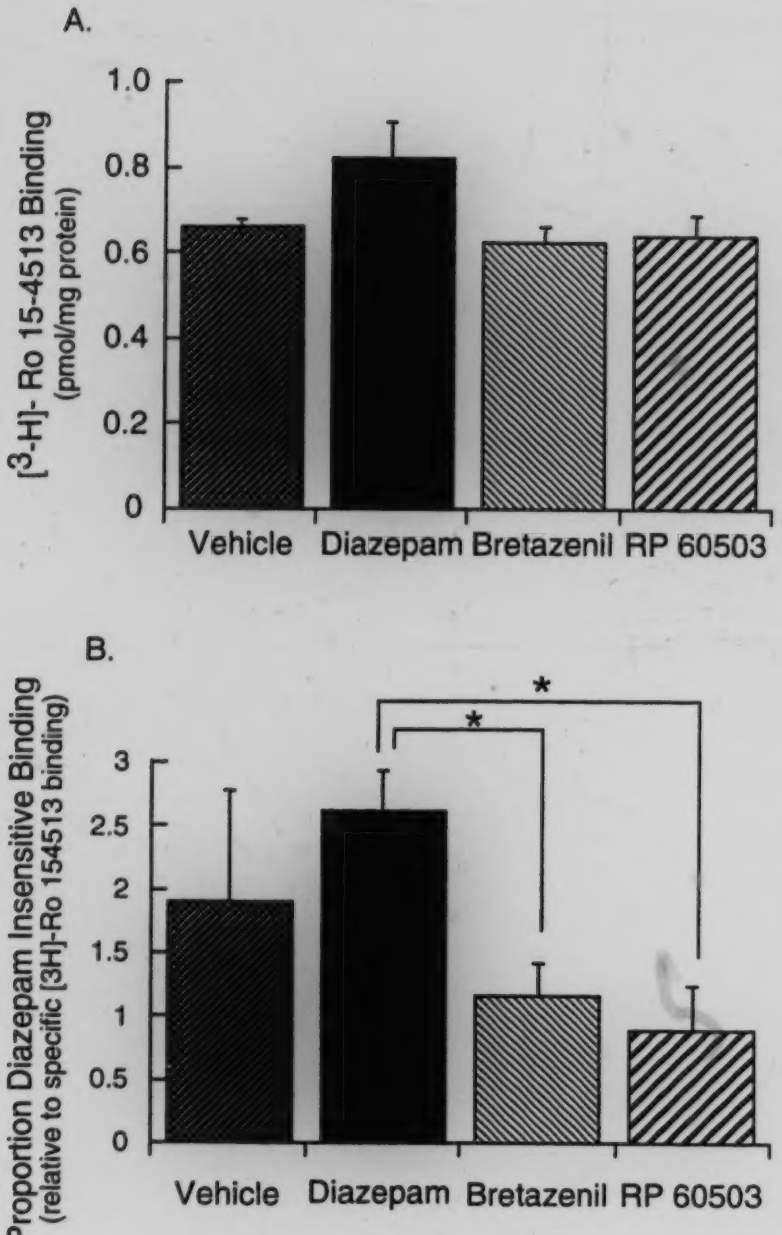
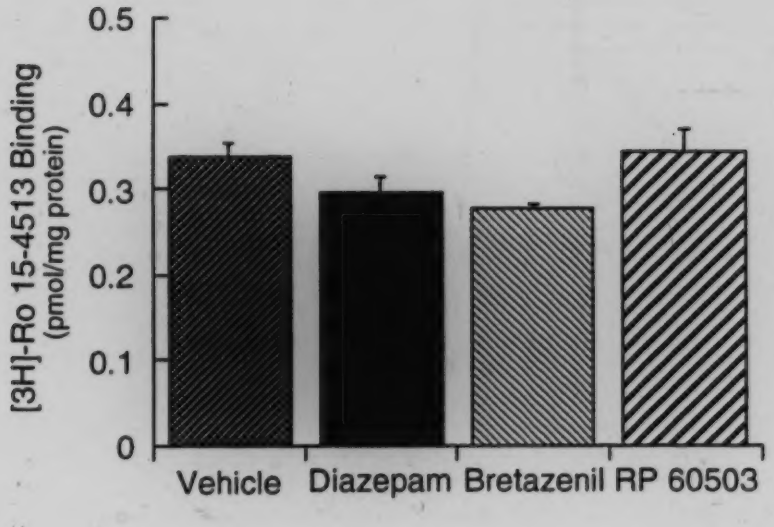

A.
[3-H]- Ro 15-4513 Binding
(pmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0
Vehicle
Diazepam
Bretazenil
RP 60503
B.
Proportion Diazepam Insensitive Binding
(relative to specific [3H]-Ro 154513 binding)
3
2.5
2
1.5
1
0.5
0
*
*
Vehicle
Diazepam
Bretazenil
RP 60503

Figure 6.7:

The mean total specific [^{3}H]-Ro 15-4513 binding (pmol/mg protein) (a) in tissue from the cerebellum of animals injected daily for 14 days with either 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatched), 7.5 mg/kg RP 60503 (large hatched), or vehicle (black hatched). Nonspecific binding was defined using cold Ro 15-4513. The mean proportion of [^{3}H]-Ro 15-4513 binding (DIS) after the addition of 10 μM diazepam (b). Error bars represent SEM calculated from binding assays done in triplicate, with n=4-6. Statistical analysis was applied using a one way ANOVA followed by a Student Newman-Keuls post ANOVA test to compare between treatment regimes.

A.


[3H]-Ro 15-4513 Binding
(pmol/mg protein)
0.5
0.4
0.3
0.2
0.1
0
Vehicle
Diazepam
Bretazenil
RP 60503

B.

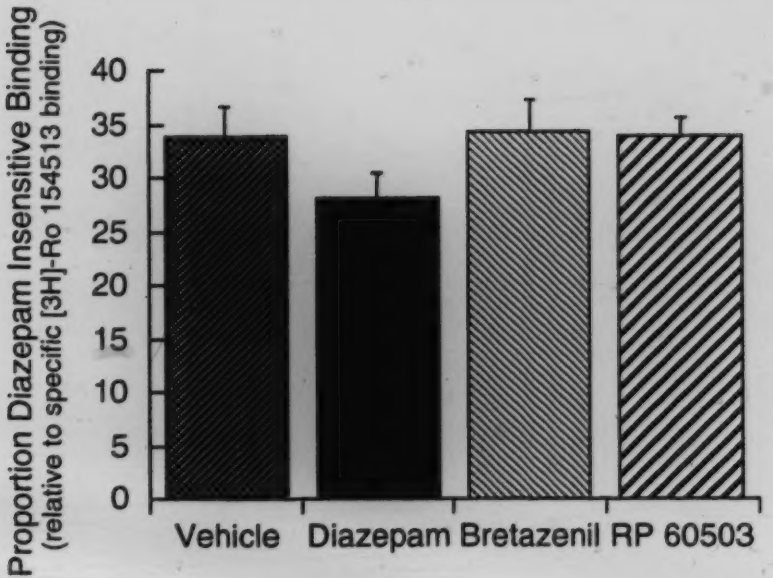
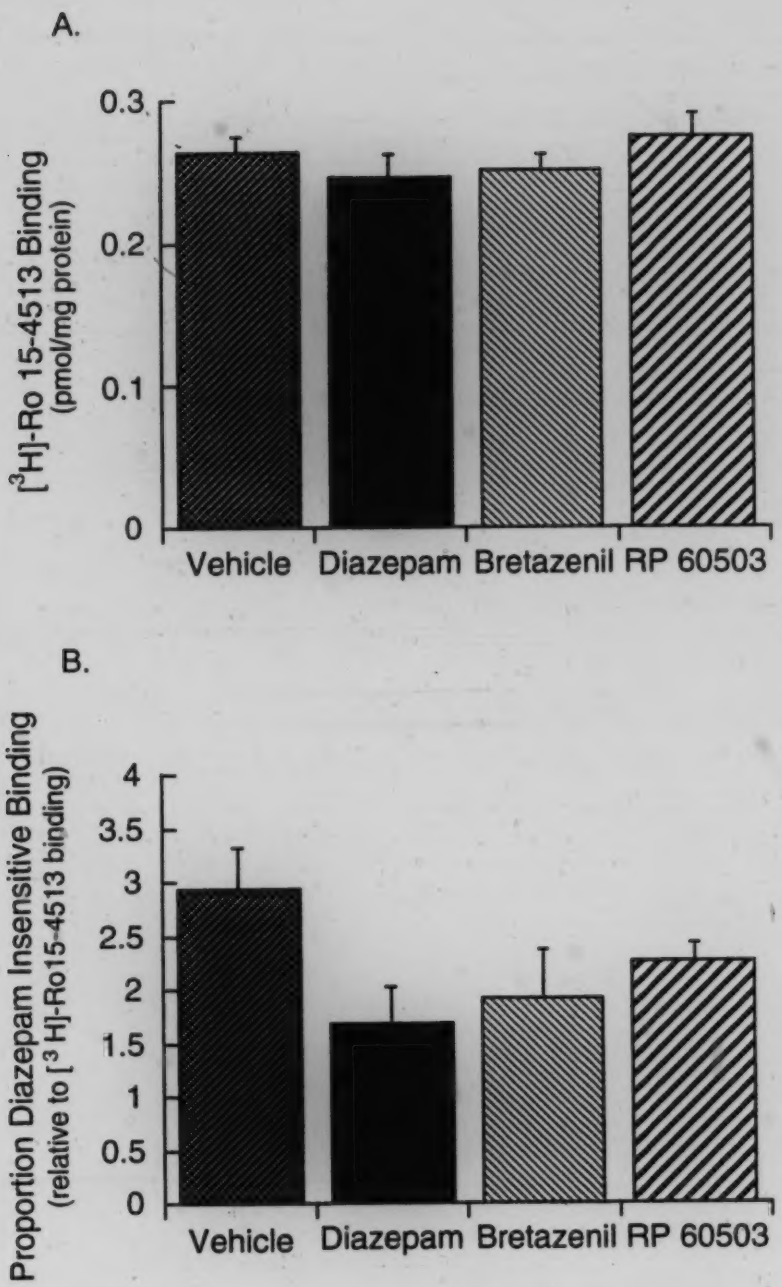

Proportion Diazepam Insensitive Binding
(relative to specific [3H]-Ro 154513 binding)
40
35
30
25
20
15
10
5
0
Vehicle
Diazepam
Bretazenil
RP 60503

Figure 6.8:

The mean total specific [^{3}H]-Ro 15-4513 binding (pmol/mg protein) (a) in tissue from the hippocampus of animals injected daily for 14 days with either 15 mg/kg diazepam (black), 0.2 mg/kg bretazenil (small hatched), 7.5 mg/kg RP 60503 (large hatched), or vehicle (black hatched). Nonspecific binding was defined using cold Ro 15-4513. The mean proportion of [^{3}H]-Ro 15-4513 binding (DIS) after the addition of 10 μM diazepam (b). Error bars represent SEM calculated from binding assays done in triplicate, with n=4-6. Statistical analysis was applied using a one way ANOVA followed by a Student Newman-Keuls post ANOVA test to compare between treatment regimes.


A.
[3H]-Ro 15-4513 Binding
(pmol/mg protein)
0.3
0.2
0.1
0
Vehicle
Diazepam
Bretazenil
RP 60503
B.
Proportion Diazepam Insensitive Binding
(relative to [3 H]-Ro15-4513 binding)
4
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
Diazepam
Bretazenil
RP 60503

CHAPTER 7

General Discussion

The $GABA_A$ receptor is an oligomeric pentamer composed of at least 3 different subunits which are encoded by separate genes; within the mammalian CNS α1-6, β1-3, γ1-3, δ, ρ, and ε have been identified. Activation of the $GABA_A$ receptor by GABA results in chloride influx, membrane hyperpolariziation and neuronal inhibition. The $GABA_A$ receptor is the site of modulation by a number of different pharmacological and therapeutic agents, and the GABAergic system has been implicated in a number of disease states: depression, agoraphobia, panic disorder and epilepsy. The modulation of the receptor system by benzodiazepines may result in changes in anxiety, sedation, seizure activity and muscle relaxation. Therefore, in a therapeutic context, the study of this receptor system *in vivo* is of primary importance. The aim of this thesis was to explore the molecular and biochemical mechanisms involved in behavioral alterations which occur as a result of full agonist benzodiazepine chronic treatment. The general hypothesis of this dissertation was that the GABAeregic system attempts to maintain homeostatic balance during chronic manipulation. In order to preserve this homeostasis, there is a certain degree of concomitant up- and down-regulation of $GABA_A$ receptor subunit expression, which in turn alters protein expression and subsequently the functional properites of the $GABA_A$ receptor.

This work has demonstrated alterations in gene expression, protein expression as measured via benzodiazepine binding capacity, and behavior as a consequence of chronic benzodiazepine exposure. In all instances, the levels of drug detected in the brain were sufficient to modulate GABAergic transmission (Guisti et al., 1993; Finn and Gee, 1993; Lavoie and Twyman, 1996). Chapter 2 established an alternative method of diazepam delivery, utilized for continuous drug infusion for a prolonged period. Further, $GABA_A$ receptor gene expression may be altered in a time dependent fashion (Chapter 3); generally these alterations in $GABA_A$ receptor subunit mRNA levels attenuate or rebound upon cessation of drug exposure. Further, these changes in gene expression were brain region and subunit specific. Chapter 4 demonstrates differential alterations in $GABA_A$ receptor

subunit mRNA levels after equivalent diazepam daily doses of 15 mg/kg/day via two distinct dosing paradigms, which produce differences in the degree of receptor occupancy. Although both treatment regimes result in compromised GABA shift, the different changes in gene expression were paralleled by differential patterns of benzodiazepine binding characteristics: primarily these dosing regimes result in altered DIS binding capacities. Moreover, the differentially altered $GABA_A$ receptor correlated to differences in the development of behavioral tolerance (Chapter 5). The dependence of changes in gene expression, however, was not limited to chronic treatment with the full agonist diazepam, as the partial agonists bretazenil and RP 60503 induced alterations in $GABA_A$ receptor mRNA levels which were also brain region and drug specific (Chapter 6). These drug induced changes were found subsequent to full or partial agonist treatment, in both $GABA_A$ receptor gene expression and binding characteristics.

The interpretation of the data presented in this dissertation relies on the $GABA_A$ receptor properties described for recombinantly expressed receptors. For instance, the properties of $\alpha 6\beta x\gamma 2$ in these *in vitro* expression models show that benzodiazepine modulation is dependent upon the exact ligand, e.g. the classical inverse agonist DMCM shows partial agonist activity at these receptor subtype combinations (Hadingham et al., 1996). However the function and modulation of this receptor subtype *in vivo* is largely unknown. In order to help interpret the data from this thesis, extrapolation from the *in vitro* properties were made in an attempt to understand and explain what the changes in $GABA_A$ receptor gene expression may accomplish *in vivo*.

Broadly examining the alterations in the α-subunit isoform in the cortex, it was demonstrated that upon chronic infusion with diazepam there was a time dependent decrease in the level of α4-subunit mRNA which returned to normal upon cessation of treatment. This temporal decrease in expression was different from the increase in the α4-subunit mRNA level after 14 days s.c. injection, and there were no significant differences between the diazepam and partial agonist treatment in the expression of this isoform after

chronic daily injection treatment. This pattern of change was similar for the α1-subunit isoform. Another pattern emerged where the changes in α5-subunit mRNA levels were similar between the two diazepam dosing paradigms but were different relative to that produced by the partial agonists. The α3-subunit isoform was different between diazepam dosing regimes and the s.c. diazepam injection was different relative to the partial agonists. Extrapolating from transient expression studies reported in the literature we know that α4-subunit containing cells have diminished binding affinity for classical benzodiazepines (Wisden et al., 1991), but partial agonists have a relatively increased affinity and efficacy at these receptor subtypes (Wafford et al., 1996; Whittemore et al., 1996). Therefore diazepam would have no effect at the α4-subunit containing receptor subtypes. The α3-subunit containing receptors require higher concentrations of GABA for maximal potentiation (Wafford et al., 1993a) relative to the α1- and α5-subunit containing receptors (Ebert et al., 1994), and the ability of diazepam to potentiate GABA-gated chloride current at the α5-subunit containing receptors has been shown to be lower than that of α1- and α2-subunit containing receptors (Puia et al., 1992).

Certain broad generalizations may be made by correlating the changes in α-subunit isoform expression with alterations in the behavioral development of tolerance. The diazepam treated animals differed with respect to anxiolytic tolerance, and there were differences between the expression of α1-, α3-, and α4-subunit genes. Further, the development of tolerance to sedation was similar between dosing regimes and the expression of the α5-subunit isoform was similar between the two diazepam groups; yet the partial agonists, which do not result in tolerance to sedative and anxiolytic effects in rats, differed in the expression of α3- and α5-subunit mRNA levels. In the cerebellum and hippocampus there was a also a significant difference in the expression of the α4-subunit mRNA levels between the partial agonists and the daily injected diazepam treated animals.

The alterations in the β- and γ-subunit were more difficult to interpret. The change from a β2- to a β3-subunit containing receptor does not appear to alter benzodiazepine

binding significantly, but it has been demonstrated that an alteration from $\alpha 3\beta 2\gamma x$ to $\alpha 3\beta 1\gamma x$ increased the GABA required for potentiation (Ebert et al., 1994). Alterations of the γ-subunit gene expression were not apparent between the partial and full agonists; however, between diazepam dosing regimes the γ1-subunit mRNA level was elevated after pump infusion, and this may result in decreased diazepam allosteric modulation of the receptor or an increase in the requirement for GABA to cause potentiation (Wafford et al., 1993c). Another possibility not examined in this thesis was the replacement of the γ-subunit class with the δ-subunit which would result in a complete loss of benzodiazepine modulation (Shivers et al., 1992; Quirk et al., 1994a).

In the cerebellum, the α1-, α4-, α5-, β1- and γ1-subunits showed differential gene expression patterns between diazepam and the partial agonists after 14 days injection, and although the magnitudes of these changes were not equivalent, diazepam infusion treatment demonstrated the same direction of gene expression alterations as the diazepam injection dosing paradigm. Changes in the hippocampus were highly variable and appeared to be more drug dependent, therefore, broad generalizations are not possible.

This shift in $GABA_A$ receptor subunit expression may further alter the number of receptor subtypes at synapses. Developmental studies have illustrated that a certain number of "embryonic subunits" are expressed during development and then subsequently the relative numbers are reduced post-natally (Poulter et al., 1992; Laurie et al., 1992; Fritschy et al., 1994). The α2-, α3-, α5-, and β3-subunit isoforms have been traced during CNS development. This work correlates with the high levels of BZ2 subtype pharmacology in the pre-natal brain (Fritschy et al., 1994), but more importantly these studies question the role of $GABA_A$ receptors in synaptic function formation. Studies have indicated that subunit genes which predominate during development are still expressed in the adult, however at lower levels. When these "embryonic genes" are expressed during development, synapses are not fully functional and only become functional after the post-natal subunit switch (Poulter et al., 1992; Fritschy et al., 1994). It is therefore possible to

hypothesize that the role of the subunits during embyronic development is not maintained after maturation. This progressive switch has been reported to be regulated by $GABA_A$ receptor activity (Poulter et al., 1997). Perhaps, after extended receptor activation by diazepam exposure, in some brain regions, $GABA_A$ receptor isoform replacement results in receptor subtypes which are not as evolved, and therefore may demonstrate different functional and cellular signaling properties.

The physiological consequences of the brain region specific changes are unknown. There was a certain degree of opposing regulation, where diazepam s.c. injection resulted in an increase in the expression of α5-subunit gene in the cortex, yet this expression in the cerebellum was significantly decreased. These changes demonstrate that the same mechanism may differentially affect a particular region's circuitry. Further, it appeared that $GABA_A$ receptor gene expression was susceptible to the overall affect of benzodiazepine chronic exposure at the GABAergic system. This was established by alterations in the expression of the α4-subunit mRNA level, as a consequence of treatment with diazepam, which has extremely low/no affinity for α4-subunit containing receptor subtypes. Therefore it may not be the drug itself which alters certain $GABA_A$ receptor subunit gene expression, but the altered modulation of GABAergic circuitry.

The sensitivity of the $GABA_A$ receptor system to modulation was also revealed by the ability of the receptor to differentially alter gene expression after administration of equivalent daily diazepam doses which differed only in the degree of $GABA_A$ receptor occupancy. For instance a hypothetical model to illustrate the temporal change at the receptor level is that with continuous diazepam infusion, 70 receptors are occupied throughout treatment, however with daily injection the number of receptors occupied ranges from 40-100 over each 24 hour period. These differential alterations of gene expression are a result of drug fluctuation or withdrawal at the receptor, which have a consequential effect on benzodiazepine binding characteristics and, subsequently, the behavioral responses in the treated animals.

The quantification of $GABA_A$ receptor protein expression, as determined by benzodiazepine binding characteristics, further demonstrated changes not only between the full and partial agonists, but between the two diazepam dosing paradigms. Based on other experiments in this laboratory where GABA enhancement has been shown to be compromised within 24 hours of benzodiazepine exposure (Holt, Bateson and Martin, unpublished), it may be hypothesized that the loss of coupling between the GABA and benzodiazepine site may an initial step in the homeostatic adjustment of the GABAergic system to deal with drug exposure. The consequence of this adjustment would be to limit the degree of $GABA_A$ receptor allosteric modulation. Further, because this alteration occurs quickly and the time course for the onset of sedative tolerance occurs within days, this may be a possible underlying mechanism for initial sedative tolerance. The partial agonist induced compromise of GABA enhancement may be the result of the different efficacies at various receptor subtypes, or may be due to the extremely high levels of $GABA_A$ receptor occupation by the partial agonists.

Changes in protein expression, as measured by the benzodiazepine binding, give an indication of what may be happening to the functional response of the receptor. Alterations in the α4- and α6-subunit were demonstrated with total [^{3}H]-Ro 15-4513 binding and the amount of DIS binding, and although not always to the same magnitude, the patterns of α4-subunit gene expression and DIS binding were similar. The difference in the degrees of change may be due to insufficient time required for protein versus mRNA level alterations, or it may be that the change in mRNA level does not produce the equivalent magnitude of change in protein levels. However, it is of interest to note the relatively uniform association of α4-subunit mRNA levels and protein expression defined by DIS binding; perhaps the α4-subunit alterations were the "rate limiting" subunit modification, and changes of other $GABA_A$ receptor subunits are dependent on this α4-subunit expression change.

The definition of BZ subtypes based on zolpidem affinity were more difficult to interpret. As previously mentioned, alterations in protein expression of subunits may not have yet had the appropriate time to change to the extent that gene expression did, or to the extent of the change being quantifiable by binding measurements. The time required for protein turnover in ion channels has been reported to vary: the minimum half-life for neuronal nAch receptor pentameric assembly has been reported to be 90 minutes, and Na^+ channel assembly takes about 2 hours (Green and Miller, 1995). Examining the kinetics of $GABA_A$ receptor turn-over in chick neuronal culture, the half-life appeared to range from 4-32 hours (for review see Rabow et al., 1995), with only about 20% of newly synthesized receptors reaching the plasma membrane (Czajkowski and Farb, 1989). Therefore the window for investigating protein level alterations must be exact, and when examining these protein levels at the same time as the altered mRNA levels were detected, protein changes may be overlooked. Furhermore, there has been no information on the time course for protein half-life in vivo, and therefore changes may be faster or slower than previously reported.

It is also important to remember that the effects of the β- and γ-subunits on BZ subtypes has not yet been fully determined. Based on the expression of α-subunit gene expression alone, parallels cannot be drawn between mRNA levels and protein levels, defined by zolpidem binding affinity. However, it is possible that there may be associations between certain isoforms, and perhaps it was alterations in the associated subunits which caused changes demonstrated by the BZ subtypes. A specific association has been reported previously between α6- and δ-subunit expression in the cerebellum (Jones et al., 1996). Extrapolation of changes in $GABA_A$ receptor β- and γ-subunit mRNA level in the role of BZ subtype binding alterations were not clear and therefore hypotheses based on their changes could not be beneficially constructed. Nonetheless, the changes, or lack thereof, in BZ subtypes demonstrate that not only are α-subunit changes important in examining $GABA_A$ receptor binding characteristics, but that these α-subunits may be

associated with alterations in other subunits which could counteract α-subunit changes in the benzodiazepine binding profile. Moreover, it was not always possible to correlate mRNA alterations with changes in binding capacity. Furthermore, partial agonist treatment altered BZ subtype binding, which may be an important illustration that drug induced alterations in mRNA levels and benzodiazepine binding characteristics may not consistently be correlated or relevant to the development of benzodiazepine tolerance.

Nonetheless, the altered/reduced sensitivity or tolerance to diazepam's behavioral effects upon chronic administration may be the response of many adaptive processes. Dispositional tolerance as a result of changes in the pharmacokinetics of benzodiazepines has been previously abandoned as a mechanism for the development of behavioral tolerance (Greenblatt and Shader, 1986; File 1982a,b; Lister et al., 1983). Pharmacodynamic mechanisms, which include both decremental and oppositional tolerance as processes of change, may be important factors in the behavioral development of diazepam tolerance. This study suggests that decremental tolerance (benzodiazepine receptor down regulation and/or decreased allosteric modulation), may act independently or may be dependent on or the result of the oppositional adaptations to chronic drug treatment. Alternatively, tolerance as a result of an adaptive learning process (Young and Goudie, 1995) may be implicated in benzodiazepine chronic behavioral tolerance (i.e. behavioral skills acquired to cope with the drug induced impairment). However it has been postulated that distinct processes may be engaged in the development of diazepam tolerance (Young and Goudie, 1995). For instance, perhaps diazepam sedative tolerance may be mediated by both learned and decremental (compromised allosteric modulation between benzodiazepine and GABA binding site) mechanisms. Anxiolytic tolerance may be mediated initially by oppositional effects which then result in decremental processes; the significant increase in α4-subunit mRNA levels, which increase the DIS binding component, result in a loss of diazepam efficacy at a subset of $GABA_A$ receptors.

In conclusion, the studies presented in this dissertation have demonstrated that chronic diazepam exposure: (1) altered $GABA_A$ receptor subunit mRNA levels in a time-dependent, brain region and subunit specific manner; (2) changed gene expression proportional to the dose-regime, where the degree of the $GABA_A$ receptor occupation differentially altered gene expression and protein levels which (3) resulted in a differential development of tolerance to behavioral measures of anxiety and (4) caused drug specific alterations in mRNA and protein levels, which were not caused by the partial agonists which do not cause tolerance. These findings are consistent with the hypothesis that chronic diazepam treatment results in a concomitant up- and down-regulation of subunit isoforms which may subsequently confer diazepam insensitivity by altering benzodiazepine binding capacity of the $GABA_A$ receptor. Further, some diazepam- induced alterations in $GABA_A$ receptor steady state mRNA levels that were not found after chronic bretazenil or RP 60503 treatment may consequently underlie benzodiazepine induced tolerance and the development of dependence.

Future Directions

The experiments included in this dissertation have answered specific questions pretaining to long term benzodiazepine administration, and have attempted to answer general questions regarding chronic treatment and the development of behavioral tolerance. If the hypothesis that drug induced $GABA_A$ receptor isoform switching underlies tolerance is correct, the best possible method to obtain proof is the use of a simplified homogenous or known cell population which is under the control of native promoters. However, such a simplified system is not yet available. The time course of drug induced changes in gene expression must further be examined at more closely spaced periods of time, and at each point monitored for corresponding alterations in protein expression. Further, commercially available or selective antibodies, or ligands that can distinguish between receptor isoforms are needed. The development of subunit specific compounds would further allow drug induced alterations at the receptor to be examined at a level beyond mRNA correlations, such as protein subunit expression, receptor trafficking, receptor internalization, and signaling pathways. In order to fully understand the relationship between tolerance development and changes in the GABAergic system the time course of behavioral alterations and changes in gene and protein expression must be implicitly examined. Henceforth, an important control experiment may be to examine if behavioral testing itself alters these expression parameters.

Knowledge about the association of subunits and the anatomical regions in which they are co-localized is slowly becoming available. It appears pertinent to examine the composition of $GABA_A$ receptor subtypes in neurons and interneurons involved in the reward pathways which have been implicated in the development of dependence and withdrawal from other chronic drug paradigms (Koob and Le Moal, 1997; Leshner, 1997; Nestler and Aghajania, 1997). Perhaps it is the $GABA_A$ receptor subtypes within these pathways that are altered to a greater degree, and the lack or smaller magnitude of changes

in gene expression throughout the gross brain regions examined may eclipse the alterations in these reward pathways.

If there is a certain degree of subunit isoform switching, or gene switching which is taking place upon chronic treatment, an obvious and important area of examination is the mechanism responsible for the signaling pathway, from the receptor to the nucleus. Previous work in this laboratory has demonstrated that not only are there similar changes in the $GABA_A$ receptor gene clusters (Holt et al., 1996), but that regulation of subunit expression can occur at the level of gene transcription (Holt et al., 1997). The experiments in this thesis have demonstrated that the $GABA_A$ receptor detects differences not only in the compounds which modulate it, but also in their mechanism of modulation. Therefore the signaling pathway for these messages must be elucidated in order to understand how tolerance to only certain receptor stimulation develops. The signaling mechanism may modulate gene expression by alterations in receptor internalization, second messanger activation, mRNA half-life, the rate of mRNA processing and transcriptional recruitment.

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[4]APPENDIX 1

Validation of the S1 Nuclease Assay

[4] Some of the data from this appendix have been published: Arnot M.I., Bateson A.N., and Martin I.L. (1996) *Br. J. Pharmacol.*. **119**:62P.

S1 Nuclease Intra- and Inter-Assay Variability

The reproducibility of the measurements of labeled oligonucleotide probes (equivalent to protected mRNA molecules) was determined by making repeated subunit isoform measurements after two seperate times. There were no significant differences between the first and the second measurements using a paired *t*-test.

Table A.1: Intra-Assay Experimental Variability of the S1 Nuclease Assay

	First measurement				Second measurement		
subunit isoform	vehicle (100%) ± SEM	bretazenil % vehicle ± SEM	diazepam % vehicle ± SEM		vehicle (100%) ± SEM	bretazenil % vehicle ± SEM	diazepam % vehicle ± SEM
α1	± 4	104 ± 2	98 ± 4		± 5	103 ± 5	102 ± 6
γ2	± 4	110 ± 10	93 ± 8		± 5	139 ± 15	93 ± 8
α1	± 3	97 ± 4	97 ±4		± 6	107 ± 7	116 ± 9
γ2	± 2	118±13	98 ± 8		± 5	125 ± 14	108 ± 3

To determine the inter-assay variability between separate experiments, the coefficient of variation was calculated for subunit isoforms α1-5, β2, and γ2 in the cortex. The % coefficient of variation was calculated from: (standard deviation/mean) x 100.

Table A.2: Inter-Assay Experimental Variability of the S1 Nulcease Assay

Subunit isoform	number of experiments	mean percentage of vehicle	standard deviation of mean measurement	% coefficient of variation
α1	5	98	5.9	6.1
α2	3	104.3	16.9	16.2
α3	4	93.5	6.6	7.1
α4	3	86.3	5.43	6.3
α5	4	100.3	4.1	3.8
β2	4	101.8	10.9	10.8
γ2	4	94	11.9	12.6

There was a certain degree of variation amongst separate experiments for several of the subunit isoforms. However, the larger variations correlated with low abundance subunit isoforms (compare α1 versus α2). Yet, all calculations of inter-assay variability were below 20%, and the majority was below 10%. Further, a one-way ANOVA was performed within each subunit class which demonstrated that there were no significant inter-assay differences between the separate experiments.

Phosphorimaging versus Autoradiographic Computer Scanning

To establish if there were differences in quantification between densitometric phosphorimaging and computer autoradiographic scanning, an experimental data set was quantified with each program, then analyzed to completion (normalized to the β-actin internal standard and reported as a percentage of vehicle control). The results were compared using a paired *t*-test. Phosphorimages were captured by exposure of the dried acrylamide gel to a BAS-IIIs Fuji imaging plate for 24-48 hours. Profiles were created using BAS 1000 MACBAS software with a Bio-imaging analyzer (Fujix) and analyzed using NIH Image software (Wayne Rasband, RSB, NIMH, NIH, Bethesda, MD). Quantification of the acrylamide gels by computer scanning took place by appossing the dried gels to BioMax X-ray film (Kodak) in an autoradiography cassette with an intensifying screen. Images were created by flatbed scanner (Hewlett Packard ScanJet 3C) and were analyzed as above with NIH Image software.

Table A.3: Differences Between Quantification of the S1 Nuclease Assay

	Scanned				Phosphorimaging		
subunit isoform	vehicle (100%) ± SEM	bretazenil % vehicle ± SEM	diazepam % vehicle ± SEM		vehicle (100%) ± SEM	bretazenil % vehicle ± SEM	diazepam % vehicle ± SEM
α1	± 6	72 ± 6	93 ± 7		± 4	76 ± 3	89 ± 5
a3	± 10	70 ± 4	100 ± 8		± 3	82 ± 2	100 ± 7
α4	± 10	144 ± 37	161 ± 61		± 6	105 ± 11	92 ± 2
β2	± 14	75 ± 8	126 ± 19		± 9	78 ± 5	103 ± 9
β3	± 12	69 ± 9	98 ± 11		± 8	75 ± 6	98 ± 7

There were no significant differences between the analysis from autoradiographic scanned data and the phosphorimage data using the paired *t*-test. However, it appears that phosphorimaging quantification yields smaller error values relative to scanned quantification. This may be the result of the higher sensitivity of phosphorimaging compared to autoradiographic computer scanning. However, both analyses gave results that were not significantly different.

Linearity and Sensitivity of Phosphorimaging

The linearity and sensitivity of phosphorimaging were determined to ensure whether the analysis was both linear and sensitive within the oligonucleotide band density and for the radioactivity for the S1 Nuclease Assay. Briefly, six oligonucleotides were radiolabeled and ten serial dilutions were made and precipitated with 15µg of carrier tRNA. These dilutions were then separated by polyacrylamide gel electrophoresis as in the S1 assay, exposed to a phosphorimaging plate (40 hours) and the image (Figure A.1) analyzed according to the methods as above and outlined in Chapter 3. The areas under the curve were measured and the background was subtracted.

The mean specific activity for each dilution of radiolabeled oligonucleotides was plotted against the mean area under the peak for the oligonucleotide bands; the SEM for each point is within the symbol. Figure A.2 illustrates the linear and sensitive relationship between the specific activity and area under the peak, or density of the oligonucleotide bands. The correlation coefficient for this curve (R^2) was greater than 0.96 and this linear relationship only slightly dropped to 0.86 when the specific activity range was increased to include $1X10^8$ (Figure A.1, lane 8). Further, the very tight error between the different oligonucleotides at the lower and higher end of this graph demonstrate that the sensitivity range was appropriate within these levels of radioactivity (Figure A.2). S1 Nuclease assay

gels were exposed to imaging plates for a time which produced a band density similar to the range seen in Figure A.1 lane 3-6.

Under the assay conditions described, there was a highly sensitive linear relationship between specific activity or radioactivity and the band intensity or density of the image generated by phosphorimaging, which is proportional to the amount of mRNA molecules protected during the S1 Nuclease Assay. Further, this Appendix has illustrated that phosphorimaging is equivalent, if not more valuable than computer autoradiographic scanning. Moreover, the analysis and measurements of these S1 Nuclease Assay gel images were consistent and the variation between experiments was minimal.

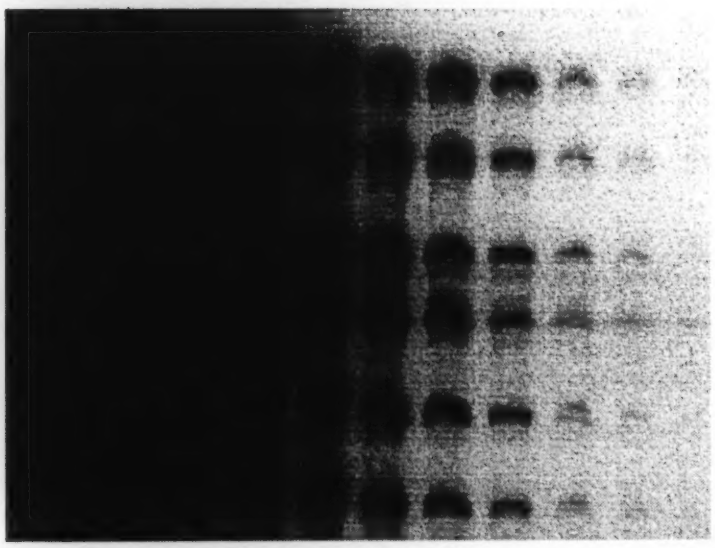

Figure A.1:

A phosphorimage of an acrylamide gel exposed for 40 hours to the imaging plate. Each lane is a serial dilution containing 6 radiolabeled oligonucleotides specific for $GABA_A$ receptor mRNA molecules. The specific activity illustrated in this figure range from greater than 10^9 (lane 10) to 10^4 dpm/mg. S1 Nuclease experiments were analyzed when oligonculeotide band darkosity was similar to that from lane 3-6.

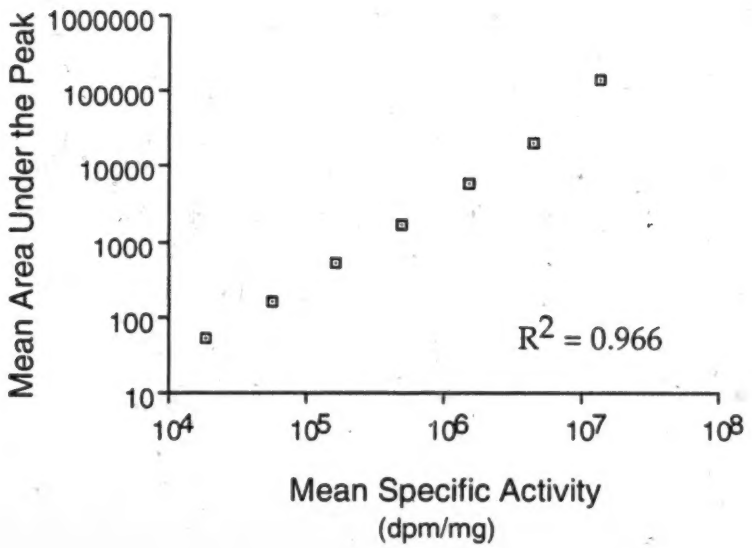

Figure A.2:

This graph demonstrates the linearity and sensitivity of phosphorimaging for the S1 Nuclease Assay experiments utilized in this thesis. Briefly, six oligonucleotides were radiolabeled and ten serial dilutions were made. These dilutions were then separated by polyacrylamide gel electrophoresis as in the S1 assay, exposed to a phosphorimaging plate (40 hours) and the image (Figure A.1) analyzed accordingly. Areas under the curve for each band were measured and the background was subtracted. The mean specific activity for each dilution was plotted against the mean area under the peak for the oligonucleotide bands; the SEM for each point is within the symbol (n=6). The correlation coefficient for this line (R^2) was greater than 0.96. This linear relationship only dropped slightly to a correlation coefficient of 0.86 when the specific activity range was increased to include lane 8 (Figure A.1) with a specific activity of greater than $1X10^8$ dpm/mg.